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**AN INVESTIGATION INTO THE EFFICACY AND MECHANISM OF
ACTION OF INSULIN AS AN AGENT FOR THE PREVENTION OR
REDUCTION OF CUTANEOUS SCARRING**

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Abstract

Cutaneous scars can be itchy, painful, disfiguring, psychologically damaging and even pathological. The myofibroblast cell phenotype, that differentiates from fibroblasts during healing, is central to the formation of normal and pathological scars. However, there remains no reliably effective therapy that prevents scarring. This research explores the efficacy and mechanism of action of a potential new antiscarring agent – insulin.

Research at the RAFT Institute of Plastic Surgery has demonstrated that insulin inhibits differentiation of skin fibroblasts into myofibroblasts in both human and mouse cell cultures and in a murine in vivo wound healing model.

The aim of this thesis is twofold. Firstly, to determine insulin's mechanism of action, in order to exploit it in future therapies. Several potential avenues were explored contemporaneously and included relatively inconclusive assessment of insulin's effects on EDA-fibronectin, FAK kinase, stress fibres and Thy-1. The most promising potential mechanism of insulin's action was its interaction with the cytokine transforming growth factor β (TGF- β) in particular the production of autocrine TGF- β and its activation. Human fibroblasts were cultured under various conditions and insulin's action investigated using techniques including immunohistochemistry, proliferation assays, multiplex and real time RT-PCR mRNA analysis, ELISA and SDS PAGE protein electrophoresis and Western blot analysis.

The second aim is to test insulin's antiscarring efficacy in humans by means of a prospective randomised controlled trial. The procedure of bilateral breast reduction was chosen as it meant patients were administered both insulin and placebo treatments therefore allowing inpatient controls. Scars were assessed 3 months

after surgery using digital imaging, clinical scar scales, panel assessments, and multiphoton microscopy analysis of moulds of the scars.

These studies have enabled progress in the development of a novel antiscarring therapy based on the antifibrotic properties of insulin.

Declaration:

The work presented in this thesis is my own.

Richard Baker

24/5/20

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Most of all I would like to thank the patients who participated in the clinical trial and hope that their altruism will one day be vindicated by the development of a successful anticarring therapy based on this research.

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List of Abbreviations

α SMA	Alpha Smooth Muscle Actin
AP	Alkaline Phosphatase
bFGF	basic fibroblast growth factor
BMP	Bone morphogenetic protein
dH ₂ O	Distilled Water
CTGF	Connective tissue growth factor
DABCO	14-Diazo dicyclo 2,2,2 octane
DEPC	Diethylpyrocarbonate
DFCS	Dialysed fetal calf serum (4% unless indicated otherwise)
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Di-methyl Sulfoxide
DTT	Dithiothreitol
DTTM	Dithiothreitol-treated dialysed fetal calf serum (4% unless indicated otherwise)
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase

FCS	Fetal Calf Serum
FGF	Fibroblast growth factor
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transporter
HRP	Horseradish peroxidase
IGFBP-5	Insulin-like growth factor binding protein 5
IGF-I	Insulin-like Growth Factor I
IGF-II	Insulin-like Growth Factor II
IL	Interleukin
IFN	Interferon
IU	International Units
JNK	c-Jun N-terminal kinase
KGF	Keratinocyte growth factors
LAP	Latency associated protein
LLC	Large latent (TGF- β) complex
LTBP	Latent TGF- β binding protein
M-6-PR	Mannose-6-phosphate receptor
MAP	Mitogen-activated protein
MLEC	Mink Lung Epithelial Cell
MMP	Matrix metallo-proteinase
mRNA	Messenger ribonucleic acid
NGM	Normal Growth Medium

OD	Optical Density
PBS	Phosphate Buffered Saline
PDGF	Platelet-derived growth factor
PI 3-kinase	Phosphatidylinositol 3-kinase
UV	Ultra-violet
RCT	Randomised controlled trial
R-SMAD	Receptor-associated SMAD
RT-PCR	Reverse transcriptase polymerase chain reaction
SARA	SMAD Anchor for Receptor Activation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
electrophoresis	
SEM	Standard error of mean
SLC	Small latent (TGF- β) complex
SMAD	Similar to Mothers Against Decapentaplegic
TBS	Tris Buffered Saline
TGF- β .	Transforming growth factor beta
TGF- β RII	Transforming growth factor beta receptor type II
TNF	Tumour necrosis factor
TRD	Total raw density
TSP	Thrombospondin
TTBS	Tween-Tris Buffered Saline
VAS	Visual analogue scale
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Introduction

This thesis continues research into the cutaneous antiscarring properties of insulin previously undertaken at the RAFT Institute of Plastic Surgery (Mackie I, MD thesis, UCL London 2004).

This introduction will discuss the events that occur during wound healing, the roles of important cells and molecules, the formation of normal scars, the aetiology of pathological scars, the current and future therapies for scar treatment and prevention and lastly the previous findings and hypotheses that underpin this work. Unless otherwise stated, the scarring and related processes discussed in this thesis are solely in reference to cutaneous scarring.

1.2 Wound Healing

A scar represents the end result of a dermal wound that has healed by a reparative process rather than regeneration. This section will discuss the healing process from the time of wounding to the formation of scar tissue. This process is divided into four merging phases: haemostasis, inflammation (or lag phase), proliferation and remodelling. Wound healing has been reviewed extensively elsewhere (Clark, 1996; Grazul-Bilska, Johnson et al., 2003) and a brief summary of these reviews is presented here with extra information cited appropriately.

1.2.1 Haemostasis

When the epidermis and dermis are breached, be it by trauma, scalpel or burn, the first event is bleeding due to rupture of blood vessels. Damage to the endothelial cells causes the local release of cytokines that increase vascular permeability and trigger the clotting cascade and the degranulation of platelets (Clark, 1996). The platelets release cytokines and growth factors such as PDGF, IGF-1, EGF, and TGF- β . These cytokines and growth factors initiate the cascades such as complement that herald the inflammatory phase and are vasoactive, being chemotactic and mitogenic with regards to leukocytes, endothelial cells and fibroblasts (Roh et al. 2006). The resulting haematoma solidifies due to fibrin formation and halts further haemorrhage. The clot has more than a haemostatic function; it also forms a temporary extracellular matrix which stimulates cell proliferation and migration. Removal of the fibrin matrix severely impairs wound healing (Clark, 1996).

1.2.2 Inflammation

This phase is initiated by haematoma formation and the release of cytokines. A series of molecular cascades ensues that result in the generation of further cytokines that act on various cells – neutrophils, macrophages, monocytes, mast cells and eosinophils to cause chemotaxis, phagocytosis, vasodilatation and local oedema (Grazul-Bilska et al. 2003). In the early inflammatory phase, neutrophils are the first inflammatory cells to reach the injured zone (first 24 hours) via the processes of margination, pavementing and diapedesis (Numata et al. 2006). These occur in response to cytokine signals particularly complement components (especially C5a), bacterial products and TGF- β (Grazul-Bilska et al. 2003). The neutrophils migrate through the tissues by chemotaxis along a cytokine concentration gradient. They begin the process of phagocytosing the dead cells and foreign microorganisms. The main cellular components of the epidermis, known as keratinocytes proliferate and begin to migrate across the wound within hours of wounding (Raja et al. 2007). They restore surface integrity and are important in restoring the basement membrane (Raja et al. 2007) that separates epidermis from dermis.

In the late inflammatory phase the neutrophils are reinforced within 48 hours by monocytes/macrophages, drawn to the wound by a number of chemoattractants including TGF- β , PDGF and complement components (Clark 1996). As well as phagocytosis the macrophages act as orchestrators of wound repair producing several growth factors that are mitogenic and that stimulate the production of extracellular matrix (Clark, 1996) and granulation tissue by other cells. Finally, after 72 hours

lymphocytes infiltrate the wound where they too are thought to play a role in collagen and ECM remodelling (Grazul-Bilska, Johnson et al., 2003). So-called “proto-myofibroblasts” also accumulate in the wound at this time. (Tomasek et al. 2002)

1.2.3 Proliferation

The proliferation phase lasts a variable period of days depending on the size of the wound and refers mainly to the proliferation of the fibroblasts and endothelial cells that form granulation tissue (Baum et al. 2005). Fibroblasts, which make up the bulk of granulation tissue, migrate into the wound from 48 hours, differentiate into myofibroblasts, and begin the synthesis of the ECM proper (collagens types I (mainly) and III, proteoglycans and fibronectin) in response to cytokines and growth factors (Darby et al. 2007). Low oxygen tension and angiogenic factors released by platelets and macrophages cause capillaries in the wound bed to sprout new capillary branches that penetrate the wound forming loops and coils and eventually re-establishing a circulation (Grazul-Bilska, Johnson et al., 2003). As the fibroblasts proliferate they start laying down collagen and the resulting mass forms the scar (Desmouliere et al. 2005).

1.2.4 Remodelling

The remodelling phase refers to the gradual maturation of the formed scar (Clark 1985). It typically takes 18 months in the adult but can take years (Mustoe et al. 2002). Essentially it represents the scar’s attempt to regain some semblance of the preinjury organisation of the dermal matrix (Mustoe 2004). This involves simultaneous break

down and synthesis of matrix components involving the proenzyme matrix-degrading metalloproteinases and enzymes collagenase, gelatinase and the stromelysins (Lorena et al. 2002). In addition collagen fibrils become reoriented so that they are parallel with the lines of tension across the wound. Modest contraction of the scar also occurs, although much less so than in other mammals such as the mouse which heal almost entirely by rapid contraction of the wound area and minimal formation of scar tissue (Tomasek et al. 2002). Remodelling is a dynamic process that requires close regulation of the balance between synthesis and degradation, disturbances in this balance lead to atrophic scars or pathological scars (Grazul-Bilska, Johnson et al., 2003). With time, myofibroblasts and small vessels disappear from the scar. It is not clear yet whether the myofibroblasts revert to quiescent forms or apoptose, but there is evidence for both mechanisms (Darby et al. 1990) (Desmouliere et al. 1995).

1.2.5 The final scar

The final scar has a tensile strength 70 – 80% of the preinjury skin (Clark 1985) and is usually paler or darker than the surrounding skin. Initially raised the scar can settle over time to become flush with the skin. The initial redness also gradually fades over weeks to months. This describes the best form of scarring, but as shall be described later, scarring can often take much more disfiguring appearances. The characteristic microscopic features of the final scar are disorganised dermal collagen architecture and absent dermal appendages such as hair follicles (Clark, 1996).

1.3 The benefits of an anti-scarring agent

Scarring causes several symptoms. Firstly, scars range in appearance from the relatively unobtrusive to the disfiguring, particularly in the case of burns and facial trauma. This in turn can lead to psychological morbidity. Children's psychological scores for happiness are inversely correlated with the number of visible scars that they have (Abdullah et al. 1994). Scars can cause low self-esteem (Robert et al. 1999) and feelings of unattractiveness which can impinge on a person's social life, work prospects and sexual relationships. The contracting nature of scars can inhibit function, particularly where they cross joints such that they can make a hand useless, prevent the proper opening of orifices such as the mouth or eyelids (Bayat et al. 2003) and can even result in growth retardation in children. An antiscarring agent could prevent or reduce the severity of these symptoms.

Although discussion so far has been confined to cutaneous scarring, scar tissue can form anywhere in the body, the only exceptions being bone, the gums, epithelial lining of the gut and sometimes the liver (Rosenthal 2005). Furthermore, the scarring process appears largely the same throughout the body. However, visceral fibrosis can lead to organ failure, death or dramatic deterioration in function such as following scarring within the lung, kidney, brain, eye or tendons. Indeed, fibrotic diseases account for up to half of the world's deaths and even greater proportion of the world's morbidities.

Thus the development of an effective anti-(cutaneous)scarring agent may also have application to other fibroses with enormous potential benefit.

1.4 The Myofibroblast

The myofibroblast is the pivotal cell in scar formation, both normal and pathological, and thus any treatments aimed at reducing scarring must directly or indirectly tackle the myofibroblast. A myofibroblast has been defined by Eyden (Eyden, 2001) as a vimentin or vimentin and actin positive cell with “prominent rER, modestly developed peripheral myofilaments with focal densities, fibronexus junctions” with “actin-filament-based junctions”. Myofibroblasts are the main cell type in granulation tissue synthesising the extracellular matrix (collagens I and III, tenascin and fibronectin) (Lorena et al. 2002). As the final scar consists mainly of collagen, preventing either myofibroblast differentiation or myofibroblast production of collagen have become the foci of much anti-scarring research.

1.4.1 The discovery and origins of the myofibroblast

The myofibroblast was discovered in 1971 by Gabbiani et al by the study of granulation tissue with electron microscopy (Gabbiani et al. 1971). It appeared then as an abnormal fibroblast that exhibited several biochemical and ultrastructural features of smooth muscle cells such as bundles of microfilaments, scattered dense bodies and gap junctions, hence the prefix myo- (Eyden 2001). These microfilaments have since been shown to be contractile fibres of smooth muscle proteins such as α -SMA, indeed there is a direct correlation between α -SMA expression and contractile activity (Hinz et al.

2003) and α -SMA remains the most reliable marker of the myofibroblast phenotype (Desmouliere 2005).

Although the myofibroblast has been characterised in detail, elucidating its exact origins has proved more challenging. Most myofibroblasts arrive in the wound from the recruitment and subsequent migration by chemotaxis of fibroblasts local to the wound in the dermis and subcutaneous tissues, perhaps via transitory “proto-myofibroblasts” (Tomasek et al. 2002). These are fibroblastic cells that accumulate in the wound in the early stages that have some morphological characteristics of myofibroblasts but express only β - and γ -cytoplasmic actins but not α -SMA (Tomasek et al. 2002). Little is known about this transition. However, other possible sources have been put forward since their discovery including macrophages (Bhawan & Manjo, 1989), muscle cells, pericytes, perivascular smooth muscle cells, circulating mononuclear cells and “fibrocytes” (Desmouliere 2005) (Postlethwaite et al. 2004).

Fibrocytes are a circulating leucocyte subpopulation with fibroblast-like properties that express α -SMA in response to TGF- β 1 and contract gels in vitro (Wang et al. 2007).

Fibrocytes enter wounds with other inflammatory cells and may be an important source of fibroblasts for the healing of extensive burns (Yang et al., 2002). Fibrocytes have been demonstrated in the hypertrophic scars of human patients (Quan et al. 2006). In analysis of tissue from mice and humans who have had sex-mismatched organ transplants, bone marrow-derived myofibroblasts have been found in several organs, although not skin (Desmouliere 2005). Lastly, during renal fibrogenesis epithelial cells have shown the capacity to differentiate into myofibroblasts in response to growth

factors including TGF- β 1 in a process called epithelial-mesenchymal transition (Postlethwaite et al. 2004).

1.4.2 Induction of myofibroblast differentiation

The development of the myofibroblastic phenotype begins with the appearance of the protomyofibroblast whose stress fibres contain only β - and γ - cytoplasmic actins and transitions into the differentiated myofibroblast with stress fibres containing α -SM actin. The induction of myofibroblast differentiation requires both cell products such as transforming growth factor β (produced by inflammatory cells and fibroblasts), extracellular matrix (ECM) components such as the fibronectin splice variant ED-A (an extracellular matrix component) and mechanical factors such as wound tension (Desmouliere 2005). In addition, several other factors have been implicated such as Thy-1, thrombin, endothelin and focal adhesions. TGF- β plays such an important role in myofibroblast differentiation and wound healing generally that it deserves its own subsection (see 1.5).

EDA-fibronectin

EDA-fibronectin is essential for the induction of myofibroblast differentiation. Fibronectin is a 440kD dimeric glycoprotein found in plasma, ECM and particularly in healing wounds (Kurkinen et al. 1980). EDA-fibronectin is one of three type III segment splice variants (Serini et al. 1998). TGF- β 1 is known to stimulate EDA-fibronectin expression in vitro, but also its deposition by fibroblasts during wound granulation precedes and is correlated with α -SMA expression both in vivo and after TGF- β 1 stimulation in vitro (Kocher et al. 1990) (Serini et al. 1998). Furthermore,

antibodies to EDA-fibronectin specifically block α -SMA fibroblast expression in response to exogenous TGF- β 1 treatment (Serini et al. 1998). EDA-fibronectin is thus a potential target for anti-scarring therapies, particularly as it is extracellular (and therefore accessible) and it is present in only low levels in (adult) tissues (potentially meaning fewer side effects if it's blocked) (Serini et al. 1998).

Wound tension

The importance of wound tension is demonstrated by the finding that the appearance of myofibroblast features including stress fibres, EDA-fibronectin and α -SMA occurs earlier in splinted (under tension) as opposed to unsplinted rat wounds (Hinz et al. 2001). Fibroblast populations in three-dimensional collagen gels demonstrate increasing α -SMA levels with increasing mechanical load (Grinnell, 2003) and even changes in fibroblast gene expression (Lorena et al., 2002). Myofibroblast contractility subsequently correlates with tension (Hinz et al. 2002). The orchestrators of the connection between wound tension and α -SMA recruitment to stress fibres appear to be “supermature” focal adhesions (Goffin et al, 2006) that transform extracellular mechanical cues into cellular responses (stress fibres) which in turn transmit contractile forces to the ECM. Furthermore and when wound contraction stops myofibroblasts gradually disappear from the wound by apoptosis (Hinz et al. 2001).

Other factors involved in the induction of myofibroblast differentiation

Besides TGF- β (see section 1.5), EDA-fibronectin and wound tension, there are other lesser factors that also may affect the induction of myofibroblast differentiation such as

cell density, cell surface glycoprotein expression and other growth factors. Corneal fibroblasts cultured at low density produce a cell culture consisting of 70-80% myofibroblasts whereas if they are cultured at much higher densities then the proportion is much closer to 10% (Masur et al. 1996). This phenomenon seems to result from the interaction between cell contact and TGF- β 1 (Masur et al. 1996).

Other growth factors also appear to play a role in myofibroblast differentiation. Y-interferon applied to cultured fibroblasts stimulates smooth muscle actin production and cell proliferation (Lorena et al. 2002). PDGF, CTGF, endothelin-1 and thrombin also stimulate myofibroblast differentiation (Desmouliere 2005).

Aside from growth factors, other forms of signalling are important too. For example, adhesion dependent signals are also crucial (Thannickal et al. 2003). Focal adhesion complexes are proteins that mediate the adhesion between the myofibroblast stress fibres and the ECM and are activated by integrins (see section 5.2 for more detail) (Goffin et al. 2006). TGF- β 1 fails to induce myofibroblast differentiation in non-adherent fibroblasts despite the preservation of TGF- β receptor-mediated signalling of SMAD-2 phosphorylation (Thannickal et al 2001). This maybe because TGF- β 1 induces phosphorylation of focal adhesion kinase (FAK) including its autophosphorylation site and this effect is dependent on cell adhesion (Thannickal et al 2001). Indeed, if FAK is pharmacologically inhibited or expressed in a mutant form, TGF- β 1 fails to induce α -SMA or stress fibre expression or even cellular hypertrophy

(Thannickal et al. 2003). Conversely, super-maturation of focal adhesion complexes requires α -SMA (Hinz et al. 2003; Hinz et al. 2003).

Thy-1 is a cell surface glycoprotein that, like EDA-fibronectin, seems to be essential for myofibroblast differentiation (Rajkumar et al. 2005). Thy-1 positive and negative fibroblasts appear distinct in terms of their production of cytokines and ECM and only Thy-1 positive fibroblasts are capable of differentiating into myofibroblasts after exogenous TGF- β 1 treatment (Koumas et al. 2003).

1.5 Transforming Growth Factor Beta

1.5.1 Introduction

TGF- β is a 25 kDa extracellular, multifunctional growth factor protein ubiquitously expressed by all mammalian cells involved in tissue injury and repair (Chen et al. 2005). It was discovered in 1983 when it was found to ‘transform’ (produce malignancy-related behaviour in non-neoplastic cells) rat fibroblasts (Roberts et al. 1983). It belongs to a group of approximately 100 cytokines known as the ‘TGF- β superfamily’ which regulate epithelial cell growth, differentiation, motility, organisation, apoptosis and tumorigenesis (Chin et al. 2004). It has important roles in wound healing, tissue fibrosis including hypertrophic and keloid scarring (for more detail see section 1.6.4) and affects extracellular matrix synthesis, degradation and remodelling (Chin et al. 2004) (Massague et al. 2000). It’s predominantly produced by T-cells but also macrophages and platelets. (Schiller et al. 2004)

1.5.2 The isoforms of TGF- β

TGF- β has three isoforms in man, 1, 2 and 3. They all equally stimulate α -SMA expression in dermal and conjunctival fibroblasts in vitro (Cordeiro et al. 2000) although TGF- β 3 inhibits myofibroblast differentiation in vivo (Lorena, Uchio et. al, 2002). TGF- β 1 accelerates wound healing whether applied topically or systemically before wounding (O’Kane et al. 1997; Chin et al. 2004). TGF- β 1 is implicated at both extremes of the scarring spectrum; since there is a very strong association between its sustained high expression and hypertrophic (Wang et al 2000) and keloid (Chin et al

2001) scarring and in addition very low and transient expression of TGF- β 1 is associated with fetal scarless wound healing (Houghton et al 1995) (Liu et al, 2004) (see section 1.7). Further, wounds treated with TGF- β 1 or 2 show more ECM matrix deposition in the early phases of wound healing whereas those treated with TGF- β 3 show reduced deposition and reduced scarring (Beanes et al. 2003). TGF- β 1 will even stimulate α -SMA production in bone marrow derived mesenchymal stem cells (Kinner et al. 2002). Both TGF- β induction of myofibroblast differentiation and its stimulation of proliferation appears to be via connective tissue growth factor-dependent pathways (Grotendorst et al. 2004). Whether TGF- β stimulates proliferation or differentiation depends on the presence of epidermal growth factor or insulin-like growth factor II respectively (Grotendorst et al. 2004).

1.5.3 The Structure and Activation of TGF- β

TGF- β 1 is secreted from cells as part of a much larger protein complex consisting of three proteins:

1. the mature TGF- β dimer
2. TGF- β pro-peptide dimer or latency-associated protein (LAP)
3. The latent TGF- β binding protein (LTBP) (Chin et al. 2004) (see fig. 1.1).

Latency of TGF- β is assured by its non-covalent association with LAP which prevents it binding to the TGF- β receptors (Chin et al. 2004). The complex of these two is called the small latent complex. The large latent complex is formed from the disulfide bonding



Figure 1.1 Schematic illustration of the a) large and b) small latent complexes of TGF- β 1 (modified from Miyazono and Heldin 1991).

between the LAP and LTBP (Chin et al. 2004). The exact role of LTBP and the purposes of its many different isoforms are unclear. Its importance is demonstrated by experiments with LTBP null mice. These animals have defective dermal matrix containing excessive amounts of fibronectin, in addition to skeletal defects, emphysema and small intestine adenocarcinomas (Annes et al. 2004). Perhaps the function of LTBPs is to ensure that TGF- β is activated in only the correct locations in tissues and the whole body, as TGF- β 's unwanted effects can be so profound and pathological (Annes et al. 2004). This hypothesis seems to be supported by the finding that transgenic mice expressing a truncated form of LTBP show premature activation of TGF- β leading to an abnormal hair phenotype (Mazzieri et al 2005).

However, the main mechanism of activation of autocrine (secreted by the fibroblasts themselves) TGF- β 1 is by dissociation of LAP from the mature TGF- β dimer (Chin et al. 2004). However, the precise mechanisms of activation and how they differ amongst the three TGF- β isoforms are unknown (Annes et al. 2002) but in essence it occurs either through interaction with the mannose-6-phosphate/IGF-II receptor (Ghosh et al. 2003) (see section 5.1.1-2) or via the action of several proteases. These proteases include plasmin and matrix metalloproteinases for example, MMP-9 and MMP-2 (Roberts, AB, 1996) (Yu and Stamenkovic, 2000). Thrombospondin 1 (TSP) is implicated in TGF- β 1 activation as TSP-1 null mice and TGF- β 1 null mice display very similar multi-organ pathologies (Crawford et al. 1998). RGD-binding integrins may well regulate activation of TGF- β 1 and 3 but not TGF- β 2 (Annes et al. 2002). Anti- α v β 5 integrin antibodies significantly impair exogenous latent TGF- β activation by

systemic sclerosis fibroblasts and reverse their myofibroblastic features (Asano et al. 2005). TGF- β can also be activated by changes in pH or temperature and exposure to oxidants (Sheppard 2005). Even radiation can activate TGF- β . (Barcellos-Hoff, 1994)

1.5.4 The TGF- β signalling pathway

Cellular responses to TGF- β requires the interaction of two distinct forms of receptor, TGF- β R type I (50kDa) and II (80kDa). Both receptors are membrane-spanning serine-threonine kinases, however type I receptors also contain a gly/ser rich domain (GS) which regulates its kinase activity. Ligand binding of TGF- β only occurs to the type II receptors which then recruit type I receptors into a membrane complex. The type II receptor then phosphorylates the GS domain of the type I receptor which activates its kinase domain. It is the type I receptor that phosphorylates the downstream signalling pathways.

The main TGF- β signalling pathway is via the SMAD pathway. The TGF- β superfamily members stimulate mRNA expression and protein production in the fibroblast by an intracellular signalling mechanism involving 'SMAD' proteins (Chin et al. 2004). These proteins are so called because of the TGF- β /BMP (bone morphogenetic protein) homologs found in the roundworm *C. elegans* ('Sma' – gene Similar to Mothers Against) and drosophila ('Mad' – gene Mothers Against Decapentaplegic) (Derynck, Gelbart et al., 1996).

There are 8 members of the SMAD family, divided into three groups on the basis of structural and functional characteristics –

1. Receptor-associated SMADs (R-SMADs) which are substrates of the TGF- β receptors. They are SMAD 1, 2, 3, 5 and 8.
2. Co-SMADs or SMAD 4 – which interact with R-SMADs.
3. Inhibitory SMADs, SMAD 6 and 7, which antagonize the action of the rest of the SMADS. (Schiller et al. 2004)

Of the R-SMADs only SMAD 2 and 3 interact with TGF- β R I. They mediate both TGF- β and activin signals. Interaction with the receptor is facilitated by SARA (SMAD Anchor for Receptor Activation) which is membrane protein that controls the subcellular localisation of the R-SMADs (Schiller et al. 2004). SMAD 2 and 3 interact directly with the ligand-activated TGF- β R I and are thereby phosphorylated on two serine residues on the receptor molecule (Schiller et al. 2004). The phosphorylated SMADs form heterodimeric complexes with SMAD4 and from here pass to the nucleus and initiate target gene transactivation (Schiller et al. 2004).

The importance of other SMADs in fibrosis has been demonstrated. For example, bleomycin-induced lung fibrosis in mice can be prevented with injection of a recombinant adenovirus carrying SMAD7 cDNA (Nakao et al. 1999). Similar results have been shown for liver and renal fibrosis (Schiller et al. 2004). Other important TGF- β signalling pathways include the mitogen-activated protein kinase pathway. In

particular TGF- β activates the extracellular signal-related kinases (ERKs) and the c-Jun N-terminal kinase (JNK) to increase the amount of SMAD 2 (Funaba et al., 2002) and stimulate fibronectin synthesis (Hocevar et al., 1999) respectively. There is evidence that scarless healing early gestation human fetal fibroblasts may behave differently to adult fibroblasts in terms of their phosphorylation of these signalling molecules in response to TGF- β 1 (Rolfe et al., 2007a).

1.5.5 Integrins and TGF- β

Integrins are transmembrane receptors that are primarily involved in cell adhesion to the extracellular matrix (Hynes 1987) but also have roles in cell signalling (Clark et al. 1995). One of these signalling roles is the binding and (pathological) activation of latent TGF- β . The integrins α v β 1, α v β 5 and α v β 6 all bind to the RGD sequences of TGF- β 1 LAP (in systemic sclerosis fibroblasts (Asano et al. 2005)) with α v β 5, α v β 6 and α v β 8 actually activating it (Sheppard 2005) (Mu et al. 2002). At least in the case of α v β 6 integrin this activation appears to be dependent on the presence of latent TGF- β binding protein (LTBP) (Annes et al. 2004) and fibronectin in the extracellular matrix (Fontana et al. 2005). Aside from integrin's role in activation of TGF- β , the expression of different integrins appears to affect fibroblast contractility and bioactivity: fetal fibroblasts with a low contractility have relatively high expression of α 2 expression and low expression of α 1 and α 3 whereas the converse is true in strongly contractile adult fibroblasts (Moulin et al. 2002). Interestingly the LAP propeptides of TGF- β isoforms 1 and 3 can act in a similar way to some ECM proteins. LAP-coated plastic supports cellular migration in a similar manner to fibronectin and is blocked by monoclonal

antibodies to $\alpha v \beta 1$ integrin. (Munger et al. 1998). Interactions with integrins of this kind may allow localisation and activation of TGF- β in on the surfaces of specific cells in precise areas.

1.6 Pathological Scarring

1.6.1 Introduction

This project seeks not only to reduce normal scarring but also the more malignant phenomena of pathological scars. These are scars that are abnormal in terms of size, confinement to wound margins, pain, pruritus, cosmetic disfigurement, duration, rate of growth, and functional impairment. Although there are a wide range of treatments for pathological scars, they are all to varying degrees, ineffectual (Niessen et al 1998) and a cure remains elusive.

There are two forms of pathological scars: hypertrophic and keloid scars, both of which are characterized by excessive dermal fibrosis. Clinically, if not pathologically, they appear to be on a spectrum with keloids at the worst end. They have distinct treatments and prognoses. They are both common. In dark-skinned individuals keloids occur in 4.5 – 16% of wounds (Cosman et al 1961 & Oluwasanmi 1974). Hypertrophic scars occur following up to 90% of deep dermal burns (Macintyre et al. 2006). Although, the clinical differences are well known, histopathologically they can look almost identical both being characterised by increased vascularity, high mesenchymal density and inflammatory cell infiltration (Lee et al. 2004). They can be defined in simple terms as follows: a hypertrophic scar remains confined to the margins of the original lesion whereas a keloid scar grows beyond these margins (Peacock, 1970). In addition, hypertrophic scars regress and infrequently recur following excision whereas keloids do not regress and recur following excision (Rockwell et al, 1989 & Pollack 1982). A

third, intermediate, group has been suggested by Muir et al (1990) that behave like keloids but remain within the original lesion's margins.

1.6.2 Hypertrophic Scars

Clinically, hypertrophic scars present as firm, red, sometimes tender lesions covered with a thin epithelium (Teot 2005). Like keloids, they show a preference for certain areas of the body, namely: thorax, neck and limbs and are less likely to occur on the ear or sole of foot (Teot 2005). Interestingly, where skin directly overlies bone hypertrophic scars do not form (Teot 2005).

The prevalence of hypertrophic scarring seems to vary amongst races. The prevalence of hypertrophic scarring of surgical incisions ranges from 15% to 63% in Caucasians and 70% in Hong Kong Chinese (Li-Tsang et al. 2005).

Histologically, hypertrophic scars can be defined as excessive masses of collagen fibrils that are confined to the original scar limits – i.e. scars with exaggerated heights (Teot 2002). The hypertrophic process begins 4 to 6 weeks after injury, expansion occurs during the 3rd to 7th months and then stabilises and the regression begins after a year (Teot 2002).

Hypertrophic scars do not form where pressure ulcers also form, but do follow wounds crossing relaxed skin tension lines (i.e. in the plane of maximum tension) or joints and other mobile areas (Teot 2005). These anatomical predilections strongly suggest that

wound tension is at the very least associated with hypertrophic scarring and indeed seems increasingly likely to be causative. They may even have a histological basis - so-called 'skin cones' (Matsumura et al. 2001). These are a normal dermal structure but are only found in the skin areas where hypertrophic scars develop (Matsumura et al. 2001).

1.6.3 Keloid scars

Keloid scars are progressive pseudo-tumoral accumulations of scar tissue extending beyond the original wound edges (Teot 2005). Dark-skinned individuals are at higher risk of keloid formation but they do occur in fair-skinned individuals too (Teot 2005). The elderly are less prone than young adults suggesting a hormonal influence (Teot 2005). Genetic factors are also believed to play a significant role, although recently TGF- β 3 was shown to have no mutations associated with keloid scarring in a cohort of Caucasian patients (Bayat et al. 2005). Nevertheless, inheritance behaves in an autosomal dominant fashion. (Teot 2005) The profibrotic TGF- β 1 and 2 are found in higher levels in keloid fibroblast cultures than normal fibroblast cultures (Polo et al. 1999) and TGF- β 1 leads to a greater increase in collagen synthesis in the former than the latter (Lee et al. 1999). Keloid fibroblasts treated with antisense TGF- β 1 meanwhile show apoptosis and inhibited proliferation (Shang et al. 2001).

1.6.4 Aetiology of pathological scars

In clinical practice the factors associated with an increased risk of hypertrophic scars are well known, namely: wound infection, tension, delayed healing, burns, youth, hereditary, poor surgical technique (Bayat et al. 2003). But the biochemical and cellular processes underlying these causes are harder to explain. Risk factors for keloid scarring seem to be more intrinsic to the individual patient such as family history and skin type. Growth factors, extracellular matrix components, abnormal collagen turnover, sebum immunoreactivity, genetic influences and tension have all been implicated but essentially the aetiology of both forms of pathological scars is unknown (Al-Attar et al. 2006).

In simple terms the pathological scars are caused by disordered regulation of wound cellularity and collagen synthesis (Sharad 2005). Abnormalities in TGF- β 1 expression, sensitivity or signalling may be involved. For example, the mRNA expression of connective tissue growth factor increases 150-fold in hypertrophic scars in response to TGF- β 1 compared with normal fibroblasts and in keloids the mRNA expression of the inhibitory TGF- β 1 intracellular signalling proteins, SMAD 6 and 7, are significantly lowered compared to normal scar fibroblasts (Colwell et al. 2005) (Sharad 2005). Furthermore, the TGF- β isoform expression profile of the dermis and epidermis seems to influence the development of normal versus hypertrophic scars in breast reduction surgery (Niessen et al. 2001).

Failure of apoptosis has been postulated as a cause for both hypertrophic scars (Nedelec et al. 2001) and keloids (Yu et al. 2005). Hypertrophic scars have fibroblasts that are highly resistant to fatty acid synthase-mediated apoptosis (Nedelec et al. 2001) and the expression of genes such as Bcl-2 that are protective against apoptosis have been shown to be elevated (Teofoli et al. 1999). Furthermore, mRNA levels of the pro-apoptotic tumour necrosis factor alpha are significantly lower in hypertrophic scars (Zhang et al. 2004). In keloids, levels of the apoptosis-involved p53 and p63 are raised (Yu et al. 2005).

There appear to be predisposing systemic traits too. Burn patients who subsequently develop hypertrophic scars have higher IL-10, TGF- β levels and elevated numbers of IL-4-positive Th 2 cells early after burn injury compared with those that don't develop hypertrophic scars (Tredget et al. 2000). 94 genes are overexpressed and 3 down-regulated in early post-burn hypertrophic scars (Wu et al. 2004). The up-regulated genes relate to proto-oncogenes, apoptosis and cytoskeletal elements among others (Wu et al. 2004). In keloids, there appears to be a major genetic contribution and compared to normal dermal fibroblasts at least 6 genes are up-regulated in keloid fibroblasts and at least 2 down-regulated (Cohly et al., 2002). A positive family history has been shown to be common in patients with keloids and is strongly associated with keloid formation in multiple anatomical sites (Bayat et al. 2005). In African-Americans it has been proposed that the increased pigmentation of their skin leads to lower vitamin D₃ levels and therefore more inflammation in response to wounding which in turn increases the propensity to pathologically scar. (Cooke et al. 2005)

Although wound tension is often cited as a cause of pathological scarring, (McCarthy et al 1990) evidence supporting this is lacking in the literature. Furthermore, a clinical study using a skin stretching device to close wounds under significant tension found that such wounds didn't inevitably form pathological scars (Melis, 2006). The severity of scarring in this study was found to be most affected by the anatomical location of the wound which reflects the varying viscoelastic properties of the skin in different areas (Melis, 2006). However, in response to tension keloid fibroblasts show upregulation of mRNA and protein expression of TGF- β 1, TGF- β 2 and collagen 1 α compared to normal (Wang, 2006).

1.6.5 Miscellaneous scars

There are other forms of scarring too that don't fall neatly into the normal/pathological scarring categories. Depressed, atrophic and spread scars occur where there is a loss of substance, particularly dermis, deep to the scar (Teot 2005) such as following a subcutaneous abscess.

1.7 Fetal Scarring

1.7.1 Introduction

Research into the scarless healing of fetuses has had a profound influence on scarring research, hence its necessary inclusion in this introduction. In 1979 Rowlatt performed a post-mortem on a still-born 20 week old human fetus that had suffered intrauterine amputations of all limbs from amniotic bands (Rowlatt 1979). She found that the amputation stumps had healed by resolution rather than by repair. It is this finding that first gave flight to the possibility of scarless healing in the adult. It has since been shown that in the first trimester the human fetal skin does not form a scar following a full thickness wound. (Chen et al. 2005) In fact the normal skin architecture including hair follicles and glands are completely regenerated. Interestingly, other tissues such as the myocardium, diaphragm, trachea and gastrointestinal tract do scar in the early fetus (Longaker et al. 2001). It has since been confirmed in other several other species too, including reptiles, rodents and sheep (Estes, 1994). The aim of fetal scarring research is to elucidate the differences between fetal and adult wound healing that enable scarless healing in the former and use this information to recreate fetal scarless healing in the adult.

1.7.2 Differences between adult and fetal scarring

At first consideration the differences between the fetal and adult wounds seem obvious. The fetal skin is bathed in sterile, growth-factor rich, nutritive fluid at a perfect 37°C – a perfect wound environment. However, sheep fetal skin transplanted to an adult will heal scarlessly outside of the womb (Lorenz et al., 1992), so the difference is not simply due to the womb environment.

Another substantial difference between early fetal and adult wound healing is the presence of a robust and mature immune system. The acute inflammatory response including inflammatory cell infiltrate is practically absent in the fetal wound. For example, fetal skin has fewer macrophages and lymphocytes compared to adult skin and any inflammatory cells present spend less time in the wound (Ren et al. 2005) (Cowin et al. 1998). This may be due to the reduced degranulation of fetal platelets (Colwell et al. 2005), reduced PDGF and TGF- β 1 and 2 content (Olutoye 1996), their reduced aggregation (Oolutoye 1997) or the reduced time the inflammatory cells remain in the wound (Cowin et al, 1998). The overall affect is a much reduced immune response to wounding. Nevertheless the finding of Lorenz et al. (1992) mentioned above and that of the opposite experiment by Longaker et al. (1994) who transplanted adult skin onto a fetus in utero (sheep) and found that adult skin still healed with a scar suggests that maturity of the immune system is not the delimiting factor. It would therefore seem that the predisposition toward perfect regeneration (fetal) or scarring is intrinsic to the tissue itself either through differences in cell behavioural programs or in local microenvironments.

Fetal and adult fibroblasts innately differ in their production of collagen (Colwell et al. 2005), hyaluronic acid (Chen et al. 1989) and other extracellular matrix components with higher and earlier levels of synthesis in the fetus. The speed of collagen production is faster in the fetal wound but the type and amounts are unchanged following wounding. Types I, III, IV and VI are synthesised and remodelled into fibrillar patterns identical to the normal dermis. In the adult wound in contrast, mainly type I collagen is produced in a disorganised pattern that forms the final scar (Chin et al, 2000). (Cuttle et al. 2005) Fetal fibroblasts also contract less in collagen gels and this may be related to the integrin expression profile (Moulin et al. 2002).

In addition, fetal fibroblasts migrate faster (Bullard et al. 2003) and many fibroblast genes have their expression up- and down- regulated as the transition to the adult phenotype occurs during gestation (Chen et al. 2006). A final telling difference is that fetal fibroblasts show less propensity to differentiate into myofibroblasts than their adult counterparts (Desmouliere et al. 1994).

More recently it has even been demonstrated that the early gestation fetal fibroblasts respond differently to TGF- β 1 than postnatal fibroblasts. Although these fibroblasts do differentiate into myofibroblasts, their response is more rapid and short-lived (Rolfe et al., 2007). They fail to exhibit a TGF- β 1-induced increase in collagen (mRNA and protein) and show a briefer phosphorylation of several components of the TGF- β 1 signalling pathways, namely Smad 2/3 and c-Jun N-terminal kinase (Rolfe et al., 2007a). Interestingly, further work by the same researcher (Rolfe et al. 2007b)

identified that these early fetal fibroblasts also failed to auto induce TGF- β 1 unlike their more mature counterparts, which produce abundant autocrine TGF- β 1 in response to TGF- β 1 stimulus. This finding raises the possibility that the tightly controlled response of early fetal cells compared to the prolonged response of developmentally mature cells is simply determined by whether TGF- β 1 auto induces its own gene transcription, thereby protracting its effect. These findings and hypotheses may explain the notable differences in the profiles of TGF- β 's in early fetal versus mature skin tissue. In human skin, gene and protein expression of TGF- β 1 is lower in early gestation compared to postnatal skin and the converse is true for TGF- β 3 (Chen et al. 2005). Indeed, in early gestation human fetal wound models, wounding does not induce TGF- β 1 mRNA or protein production (Nath et al. 1994; Lin et al. 1996). Interestingly, the mRNA expression of TGF- β 2 meanwhile is increased in early gestation although actual protein expression increases rather than decreases throughout gestation (Chen et al. 2005).

Although it produces very little TGF- β 1, fetal skin retains the ability to respond to TGF- β 1 – a TGF- β 1 impregnated implant will induce adult-like scarring in terms of fibroblast proliferation and collagen accumulation in fetal rabbit skin (Krummel et al. 1988). TGF- β 1 may induce scarring in normally non-scarring skin by increasing ECM production and decreasing its degradation. This is supported by the finding that addition of exogenous TGF- β 1 decreases human fetal collagenase expression (Bullard et al. 1997) and upregulates collagen I gene expression (Gallivan et al. 1997). Furthermore, reducing TGF- β 1 and 2 levels in adult wounds with antibodies, antisense oligonucleotides or synthetic antagonists inhibits subsequent scarring (Shah et al.

1995). The differential expression of the TGF- β isoforms may allow for a spectrum of wound healing responses from antiscarring to profibrotic depending on the stage of gestation (Chen et al. 2005).

The TGF- β receptors have also been hypothesised as playing a role in fetal scarless healing, since the gene and protein expression of both receptors I & II (TGF- β RI and TGF- β RII) is reported to be lower in early gestation skin compared to postnatal skin (Chen et al. 2005). Thus, early gestation skin may have a lesser ability to detect and respond to the TGF- β ligands and therefore permit a less fibrotic reaction to wounding (Chen et al. 2005). These observations would suggest that the complete inhibition of TGF- β 1 action or myofibroblast induction is not necessary to achieve scarless healing but rather their curtailment.

The discovery of fetal scarless healing has led to many of the ideas driving current anti-scarring research and inevitably influences the work contained in this thesis. It remains to be seen whether this direction does ultimately lead to the cure for scarring.

1.8 The clinical management of scars

1.8.1 Introduction

The management of scars is a centuries old basic tenet of surgical practice. It begins with prevention of poor or pathological scarring with good surgical technique and careful wound care. Irrespective of this a wide spectrum of scarring severity occurs, thus treatment of scars remains a common clinical problem, and as yet one without a satisfactory answer. Clinical and/or assessment of established normal and pathological scars is essential both for judging the efficacy of new scar therapies but also for monitoring the progress of patients in the surgical outpatients clinics. These issues are particularly important in the context of this thesis in order to choose the best method for assessment of the clinical trial results.

1.8.2. Assessment of scars

The purpose of scar assessment is to detect abnormal scarring early and monitor response to treatment in a standardised reproducible way (Teot, 2002). If scar assessment is adopted in the first three months after healing, warning signs of pathologic scarring such as excessive erythema may be detected. Appropriate treatment can then be instigated in an attempt to prevent the establishment of treatment resistant scars (Teot 2002).

Histopathological examination is the gold standard for scar assessment, now aided by digital image analysis. The thickness of the dermis, the degree of inflammatory infiltration and type and orientation of collagen fibrils (Rawlins et al. 2006) can all be measured but the invasive nature of acquiring this data is not appropriate in many clinical scenarios, thus scar rating scales (of which there are currently at least nine in the literature) have been developed that facilitate objective assessment of scars without the need for biopsy (Masters et al. 2005).

1.8.3 Clinical scar assessment scales

Assessing scars in the clinical setting is an area fraught with inconsistencies and lack of objective measures. Scars are hugely variable. They are influenced by the size of the wound, the mechanism of wounding, the accuracy of repair, infection, skin type and pathological scarring. Even if all these factors are controlled for, scars remain notoriously difficult to objectively compare. The reasons for this are twofold: firstly, scars do not have any easily quantifiable feature and secondly the properties of a scar that influence our subjective judgement of its severity are multiple. These include: colour, prominence, size, pathological type, anatomical location, orientation, edge and regularity. In addition, the patient with a scar may add several more properties: pain, itching, tightness, inhibition of function (for scars crossing joints). Thus clinical methods used to assess scars tend to include several parameters, each given a score and then combined to give an overall score. These methods are referred to as 'clinical scar scales'.

The first scar scale, the Vancouver Burn Scar Assessment Scale, rates scars on pigmentation, vascularity, pliability and height (Sullivan et al. 1990). It has since been modified to make clinical use easier (Baryza et al. 1995). It has high interobserver correlation and patient satisfaction scores show significant correlation with Vancouver scores of mastectomy scars (Truong et al. 2005).

However, there are a number of problems with this scale. Firstly, it is difficult to separate the effects of pigmentation and vascularity, and secondly pigmentation values are not ordinal and therefore cannot be added to the remaining values (Zuijlen et al, 2001). Furthermore, the human eye is unable to quantify relative intensities of brown and red – it simply perceives one colour (van Zuijlen et al. 2002). Scar contour, which can vary enormously, is not rated (Truong et al. 2005). Furthermore, the scale is specifically designed for burn scars and hence is not suitable for assessing surgical scars although it was found useful in assessing breast cancer surgery scars when combined with short-form McGill Pain questionnaire and patients' scar ratings (Truong et al. 2005). Because of these limitations to the Vancouver scale, the concept has been further developed to make descriptions numerical; to include scar location and patient observation, and to broaden the application to linear non-burn scars (Masters et al. 2005) (Draaijers et al. 2004) (Beausang et al. 1998).

Masters et al. addressed the inevitable problems with interrater variability and, in particular, difficulty in rating colour/pigmentation by adding reference photos to a modified version of the Vancouver scale. (Masters et al. 2005) The patient and observer

scar assessment scale has separate scoring systems for both the clinician and patient thereby taking into account not just a scar's physical properties but also the symptoms associated with it. Interestingly, the validation study for this scale found that the properties of a scar that contributed most to patients' overall assessments of their scars was itchiness and thickness in contrast with the factors that most influence the physicians: vascularity, thickness, pigmentation and relief (Draaijers et al. 2004). Like the Vancouver scale, the patient and observer scale was designed for rating burn scars, however it has also shown good intra- and interobserver correlation for surgical linear scars (van de Kar et al. 2005).

Finally, the Manchester scar scale is an observer scale that shows good intra- and interobserver reliability and was designed for assessing scars of all aetiologies (Beausang et al. 1998). Unlike the Vancouver scale this scale scores a scar's characteristics not in absolute terms but in terms of their contrast with the surrounding skin. The characteristics it scores are: colour (combining pigmentation and vascularity), shiny/matte, contour, distortion and texture (Beausang et al. 1998). To further separate scars the scale includes a visual analogue scale used to grade the overall appearance of the scar. This is converted to a score from 1 to 10. What's more, the scores this scale gives various scars have been shown to correlate well with the histological assessment of scars (Beausang et al. 1998).

1.8.4 Objective assessment of scars

Scar scales, however well crafted, are inevitably limited by degrees of subjectivity. An affordable non-invasive objective scar-quantifying device would circumvent this problem and may soon be a reality as several properties of a scar can already be objectively measured. These include, pigmentation (via digital image analysis), thickness (via ultrasound), surface area and texture (via digital photography and optical or mechanical profilometers) (Niessen et al. 1998).

Scar colour is a combination of red (vascularity and erythrema) and brown (melanin hyper- and hypopigmentation). A colour model based on human perception that seeks to objectively separate these two colours has been developed for dermatological research (Westerhof, 1995). However, there are now devices that can measure the individual tri-stimulus colour values such as the Minolta Chromameter (Draaijers et al. 2004). Other devices such as the DermaSpectrometer® can separate and measure the erythrema and melanin indices using image analysis software (Takiwaki et al. 1994).

Measurement of surface area (planimetry) can be achieved most simply using tracing paper and marking the scar margins or, and more accurately, using digital photography. Technological methods involving scanning and computerised 3-D reconstructions and body scanners are also available, although very costly (Mekkes et al. 1998) (Wood et al. 1996) (Powers et al. 1999). The surface irregularity of scars can be quantified with optical or mechanical profilometers (Fischer et al. 1999) (Quan et al. 1997).

The thickness of burn and breast reduction scars has been reliably measured with ultrasound. It has the advantage of being relatively cheap but the disadvantage of being operator-dependent (Tan et al. 1982) (Fong et al. 1997) (Niessen et al. 1998). Magnetic resonance imaging has been used for measuring skin, though not scar, thickness (Richard et al. 1993). Scar volume can also be accurately calculated with experimental 3-D imaging technologies (Rawlins et al. 2006).

Scars have several mechanical properties but the most important in terms of symptoms is scar elasticity. This is because it is inelasticity and contraction of scars that cause painful tension and distortion of surrounding skin and impair the movement of joints or distort anatomical openings. Scar stiffness is related to tissue organisation which reflects the orientation of collagen fibrils which can be determined by measuring the velocity of sound wave propagation through scar tissue (McHugh et al. 1997). Scar elasticity has been measured successfully with non-invasive suction devices such as the Cutometer[®]; pressure devices such as the cicatrometer, and torque devices such as the Dermal Torque Meter (Boyce et al. 2000; van Zuijlen et al. 2000). Skin, but not scar, elasticity has also been measured using a pressure device, the Durometer (Falanga et al. 1993).

Finally, scars can also be assessed in terms of how they affect function. For example, if a scar crosses a joint then the range of motion of that joint can be measured with goniometry and compared with the same joint on the opposite side. Alternatively, the effects of scarring can be measured in terms of their effects on overall activities of daily

living (useful for patients with large burns) or hand function if relevant (Badley et al. 1987) (van Lankveld et al. 1996).

1.9. Treatment of scars

1.9.1 Introduction

There are many treatments available for a scar once formed. They range from the physical to the pharmaceutical to the surgical. However, no current therapy will make a scar revert to normal unscarred skin. Evidence supporting the use of many of these treatments is poor. Indeed, the authors of the “International clinical recommendations on scar management” felt that the only treatments that had enough supporting evidence to make an evidence-based recommendation for their use were silicone gel sheeting and intralesional steroid injections (Mustoe et al. 2002). But even these treatments have their drawbacks. Prolonged silicone treatments help to flatten and soften hypertrophic scars but do little for normal or keloid scars whilst steroid injections if repeated too often are lead to thinning of the dermis, telangiectasia, pigmentation changes and in children possible adverse growth effects. Thus the search for an effective anti-scarring remains a pressing clinical need.

1.9.2 Physical therapies

Silicone gel sheeting remains first line treatment for normal and hypertrophic scars and has been proved efficacious in a large metanalysis (Poston, 2000). Silicone gels have demonstrated improvements in redness, itching, texture and thickness of hypertrophic scars in 60% to 100% of cases across a range of trials (Lorenzo, 2002) although this improvement is not complete. The mechanism of action remains unclear, but it has been

suggested that they work by hydrating scar tissue or even by generating static electricity.

Massage may be effective for softening scars although it doesn't actually reduce scar volume (Bayat et al. 2005). Massage is widely practised for scars although evidence for its use is anecdotal no prospective randomised controlled trials having been performed to address this issue (Roques, 2002). The efficacy of massage is probably due to a reorientation of collagen fibrils (Katz, 1992). Moisturising lotions that are promoted as scar treatments probably owe their limited success to the massage that accompanies their use.

Pressure therapy in the form of compression garments and hydrotherapy are widely used particularly for hypertrophic burn scars. However strong evidence for their efficacy is lacking (Mustoe et al. 2002) and the drawbacks to the use of pressure garments are well documented (Macintyre et al. 2006). This is particularly unpleasant treatment requiring the wearing of an uncomfortable, hot garment for a minimum of 23 hours per day, for eighteen months. Furthermore, young children are often the patients that need this treatment the most.

A number of lasers are in use for the treatment of scarring although evidence of efficacy is again largely anecdotal (Mustoe 2004). Pulsed-dye lasers may be useful in treating resistant keloids in combination with intralesional steroids (Kuo et al. 2005) (Mustoe et al. 2002). Laser-treatment may also flatten hypertrophic scars and reduce

erythema although with conflicting reports of success (Smit et al. 2005). So called 'laser welding' of skin wounds seems to produce better scars in rats (Gulsoy et al. 2006).

Cryotherapy tends to be limited to the treatment of very small scars because of the attendant side-effects of pigmentation changes, skin atrophy and pain. However, a method of delivering intralesional cryotherapy using a needle attached to a liquid nitrogen source has been described which appears to be particularly effective at shrinking keloid scars (Har-Shai et al. 2006). Keloids can also be treated effectively with radiotherapy in combination with surgery but carries a significant risk of carcinogenesis (Al-Attar et al. 2006).

Other physical therapies include ultrasound, static electricity, and pulsed electrical stimulation. These are as yet unproven by randomised controlled trials (Mustoe et al. 2002)

1.9.3 Medical therapies

Intralesional injection of corticosteroids, usually triamcinolone, is the most commonly accepted medical treatment of pathological scarring (Mustoe et al. 2002). Steroids work by dampening the inflammatory response and as such are only effective in "active" scars but not in normal quiescent scars. Steroids may reduce the size and symptoms of pathological scars but do not eliminate them. They are most effective in the treatment of keloids rather than hypertrophic scars, particularly when combined with other

modalities such as excision, pressure therapy and silicone sheeting/gels (Berman et al. 1996). Steroids have been shown to be effective for both types of pathological scars in terms of recurrence (Giovannini, 2002). For example, keloids treated with excision and triamcinolone injection demonstrated 19% recurrence versus 51% recurrence with excision alone (Berman et al. 1996). Triamcinolone stimulates basic fibroblast growth factor (bFGF) production, inhibits collagen synthesis and decreases cellular proliferation. (Carroll, Hanasono et al. 2002) However, steroids do not improve normal scars and have several disadvantages: intralesional injection is intensely painful, skin changes such as thinning, depigmentation and telangiectasias, and the growth of children can be inhibited with repeated doses.

Interferon has been reported to increase collagen breakdown, improve hypertrophic scars and prevent recurrence of keloids better than triamcinolone (Mustoe et al. 2002). However, topical imiquimod (interferon 2 α inducer) and interferon 2 α b have both recently been found to be ineffective in the treatment of normal scars and keloids respectively (Berman et al. 2005) (Davison et al. 2006). The cytotoxic cancer chemotherapy drugs 5-fluorouracil and bleomycin have shown potential efficacy in flattening hypertrophic and keloid scars (Kontochristopoulos et al. 2005) (Saray et al. 2005). However, all these agents require investigation within larger RCTs (randomised controlled trial(s)) before general conclusions can be made.

Other researchers are approaching the problem more directly via the myofibroblast. Desmouliere et al have found that the N-terminal sequence of α -SMA (AcEEEEED)

inhibits α -SMA incorporation into stress fibres thus reducing the tension produced by the fibres and is associated with a significant decrease in collagen production (Desmouliere et al 2005). They suggest other possible methods too: inhibiting myofibroblast apoptosis and/or replication or inhibiting collagen and proteolytic enzyme production (Desmouliere et al 2005).

Verrechia et al have concentrated on the main intracellular signalling pathway of TGF- β -SMAD proteins (see section 1.5.4) and they found that a transcription factor Sp1 interacts with SMAD proteins to activate several genes in response to TGF- β and is also important in permitting the expression of collagens. Thus they hypothesize that altering the function of Sp1 may yield anti-scarring effects. They have shown that targeting of the Sp1 gene broadly inhibits ECM gene expression in fibroblasts and that decoy Sp1-binding oligonucleotides inhibit COL1A2 promoter activity *in vivo* in mice (Verrechia & Mauviel 2002). Furthermore, signalling proteins such as RelA and cJun can inhibit SMAD-driven promoter transactivation by competing with SMAD for p300 (Verrechia & Mauviel 2002). Verrechia et al suggest other avenues of research such as promoting the over-expression of the inhibitory SMAD 7 and manipulation of connective tissue growth factor since it mediates TGF- β 's effects on ECM deposition (Verrechia & Mauviel 2002). However, development of these scientific observations into a clinical solution still requires substantial work.

Perhaps the most promising potential medical therapy stems from the research into fetal scarring and TGF- β . In particular, lowering the levels of TGF- β 1 and 2 in rat wounds

with neutralising antibodies infiltrated into the wound margins leads to reduced macrophage and monocytes profiles, reduced neovascularisation and reduced fibronectin, collagen I and III deposition compared to control wounds (Shah et al 1995). The architecture of the neodermis is also markedly improved such that it resembles normal dermis. Furthermore, addition of exogenous TGF- β 3 or mannose-6-phosphate (competitive inhibitor of TGF- β activation) to the wounds had the same effects (Shah et al 1995). Interestingly wounds treated with TGF- β 2 neutralising antibodies alone did not show an improvement in scarring (Shah et al 1995). These findings have led to testing of new agents that are now undergoing phase II clinical trials (Ferguson et al. 2004).

TGF- β can be targeted in other ways and some of these are suggested by research in other disciplines. For example, TGF- β is the prime cytokine in alcohol-induced liver fibrosis. Some successful anti-TGF- β approaches to experimental liver fibrosis include mutant T TGF- β RII, camostat mesilate (a protease inhibitor), TGF- β binding proteins such as decorin and antagonistic cytokines such as bone morphogenetic protein-7, hepatocyte growth factor, IL-10 and IFN- γ (Breitkopf et al 2005).

The central role of TGF- β 1 in scarring suggests targeting the molecule and its receptors is the obvious approach to reducing scarring. However, TGF- β 1 has a multitude of other functions which could also be affected. For example, TGF- β 1-null animals develop multi-organ autoimmune inflammation (Shull et al., 1992) and transgenic mice with kinase-deficient TGF- β receptors type II ligand-dependent develop paradoxical

activation of TGF- β signalling causing skin and lung fibrosis. Indeed, TGF- β 1 is also known to induce migration of epidermal keratinocytes thus blocking it completely could feasibly delay wound closure.

Other antiscarring therapies under investigation include pentoxifylline, prolyl-4 hydroxylase (digests collagen), verapamil (calcium channel antagonist), tacrolimus and anti-TNF- α agents (Rawlins et al. 2006) (Kim et al. 2003) (Copcu et al. 2004) (Kim et al. 2001). Apart from those indicated, these are all essentially anti-inflammatory drugs and thus dampen the production of cytokines that drives the scarring process. However, these are all experimental and as yet untested by large RCTs therefore they will not be described in detail here. Finally, there also exist a number of homeopathic remedies for scarring but these have not had their mechanisms of action investigated or their efficacies tested by clinical trials and thus these too will not be further discussed.

1.9.4 Gene therapy and scarring

In principle, gene therapy could be applied to scar prevention. For example, TGF- β 1 expression would be a potential target (see section 1.5). The first attempt to manipulate TGF- β 1 at the genetic level was by Choi et al who injected antisense TGF- β 1 oligonucleotides into mouse wounds and found improved scarring compared to those injected with sense TGF- β 1 oligonucleotides. (Choi et al. 1996) Genetic manipulation of the ratios of expression of the pro- and antifibrotic isoforms of TGF- β is a further possibility.

Endogenous TGF- β 1 gene expression can be reduced by transfecting fibroblasts with a truncated TGF- β R I gene (Liu et al. 2004) or a truncated TGF- β R II (in mink).

(Yamamoto et al 1996) In keloids the same approach has led to decreased TGF- β expression and inhibited cell proliferation (Liu et al 2002). TGF- β signalling can be indirectly targeted via individual SMADS. Transfection with antisense SMAD 3 and sense SMAD 7 down-regulates TGF- β expression. (Liu et al 2004) SMAD 3 knock-out mice show accelerated reepithelialisation of wounds out whilst transfecting additional full length sense SMAD 7 cDNA leads to a downregulation of TGF- β 1 expression (Ashcroft et al, 1999) (Liu et al. 2004).

However, scarring has yet to be subjected to the amount of gene therapy research as other areas so at present the clinical use of antifibrotic gene therapies probably remains a distant prospect (Liu et al. 2004).

1.9.5 Surgical Advances in Scar reduction and revision

The severity of postoperative scarring can be minimised with meticulous surgical technique. Careful tissue handling, leaving behind as little foreign material as possible and avoiding infection by thorough antisepsis will reduce the inflammatory response. The incision should be placed parallel to the lines of relaxed skin tension which will decrease the ‘stretching’ tension on the final scar.

Surgical technology is contributing to improved technique. For example, using glue (octylcyanoacrylate) in place of skin sutures may produce a better scar in head and neck surgery and paper tape has repeatedly shown efficacy in reducing the incidence of hypertrophic scarring particularly if applied for a prolonged period (12 weeks) (Laccourreye et al. 2005) (Reiffel 1995; Atkinson et al. 2005). The so-called “surgical zipper” technique has also been tested in a fifty wound trial and reported as significantly improving subsequent scars (Onuminya et al. 2006).

If conservative and medical measures fail to sufficiently improve a scar, then revision surgery may be necessary. The traditional methods of re-excision plus adjuvant therapy, Z- and W-plasties, grafts and flaps remain the mainstay but newer techniques are now available such as dermabrasion, chemical peels and follicular unit micrografting (Westine et al. 2005).

Other advances have focused on reducing wound tension. One method avoids tension by leaving the dermal element of the scar unexcised and closing epithelial skin flaps over the scar. Thus the scar continues to take up the tension in the dermis but any widening is very effectively hidden (Wilson 2000). The similar 'fillet flap' has been described for revising keloid scars. The skin over the keloids is raised as flaps, the keloid tissue excised and the skin resutured thus closing the wound without tension and without a skin graft donor site. (Kim et al. 2004) Alternatively, after the keloid tissue has been excised, a split thickness skin graft can be applied to the bed. Wound tension is avoided and the skin graft naturally contracts over time. (Saha et al. 2004)

Unfortunately, all of these techniques result in a further scar with a high chance of recurrence of pathological scars (50% to 80% for keloids (Darzi et al., 1992)). There is also an inevitable risk of complications such as wound infection, wound dehiscence and anaesthetic problems.

1.10 Insulin and scarring

1.10.1 The discovery and nature of insulin

Insulin was discovered in 1921 and was one of the greatest medical advances of the twentieth century. Insulin's discovery heralded a great leap forward in morbidity and mortality of diabetics. Banting and Macleod received the Nobel Prize in 1923 for their discovery. Insulin also enabled Fred Sanger to win the Nobel Prize for Chemistry in 1958 by being the first protein to have its amino acid sequence determined. Insulin is a 5.8kDa protein hormone with profound and broad effects. It is mitogenic in many cell types and suppresses apoptosis in neuronal cells, endothelial cells and fibroblasts (Stout et al. 1990) (Hermann et al. 2000). Surprisingly however, the exact mechanism of the main action of insulin still remains unclear 50 years later (Cohen 2006). Bracknell Levine in 1949 theorised that insulin lowered the amount of glucose in the blood by facilitating the transmembrane transport of glucose in to various extra hepatic tissues (Levine R 1949) and this is now known to be achieved with the glucose transporter 4 (GLUT4), which is expressed uniquely in insulin-sensitive muscle and adipose tissue (James et al. 1988; Birnbaum 1989). How insulin stimulates this translocation is only now being unravelled but its receptor has been identified and is a protein tyrosine kinase. This mediates insulin signalling by phosphorylating a number of proteins including insulin receptor substrate 1 which acts as a secondary messenger to activate insulin related genes (Prisco et al. 1999).

1.10.2 Insulin and scarring

Although a connection between insulin and cutaneous scarring has not previously been proposed in the literature, evidence of a potential link with fibrosis of other tissues is well described. Diabetes mellitus and the milder condition of insulin resistance are both characterised by widespread fibrosis throughout the body tissues. Chronic diabetics suffer from non-alcoholic steatohepatitis, diabetic kidney disease, atherosclerotic plaque formation and pancreatitis, all manifestations of inflammation and fibrosis (Berria et al. 2006). Even the skeletal muscle of insulin-resistant people has elevated collagen content (Berria et al., 2006). Non-diabetic burn patients administered with systemic insulin demonstrate faster healing of skin graft donor sites (Pierre et al. 1998) and topical insulin administered to incisional skin wounds of non-diabetics also heal more quickly (Greenway et al. 1999) although unfortunately in neither of these studies is the effect on scarring recorded.

There are also hormones other than insulin that are known to have antifibrotic effects. Growth hormone inhibits TGF- β -induced myofibroblast differentiation leading to reduced fibroblast contractile activity in humans and in transgenic mice over-expressing growth hormone, excisional wound closure is delayed (Desmouliere 2005). Therapeutic steroids have well known antifibrotic and scar resolving actions.

Insulin and TGF β -1 also have a loose connection via glucose: high ambient glucose appears to increase TGF- β 1 sensitivity in human mesangial cells (in the kidney). Specifically mesangial cells exposed to high glucose concentrations (20mM) compared

to lower levels (6.5mM) show higher R-SMAD phosphorylation and nuclear accumulation and express higher levels of type I collagen mRNA expression and promoter induction (Hayashida et al. 2004). Furthermore, this effect is abrogated by a mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK inhibitor (Hayashida et al. 2004). There are even examples of insulin having antagonistic effects to TGF- β 1. In human corneal epithelium insulin promotes proliferation and inhibits apoptosis and TGF- β 1 has exactly the opposite effects (Yanai et al. 2006).

This relationship has been previously studied indirectly using diabetes mellitus impaired wound healing models. These models are characterized by decreased granulation tissue, reduced amount of collagen protein and DNA and delayed wound healing (Hunt et al. 1976; Seifter et al. 1981). However, addition of TGF- β can generate increases in the accumulation of granulation tissue (Broadley et al. 1989) and collagen content of these wounds and partially reverse the reduced tensile strength of diabetic incisional wounds (Bitar et al. 1996). Furthermore, insulin applied to wounds in diabetics accelerates wound healing (Servold 1991) although the effect on the subsequent scarring in these patients was not recorded.

1.10.3 The myofibroblast inhibiting action of insulin – current knowledge

Research by Dr C. Linge and her team at the RAFT Institute (personal communication, C. Linge) has produced new findings that describe insulin's anti-fibrotic properties. These findings are the foundations on which this thesis is based. Human dermal

fibroblasts cultured in growth-factor deactivated (GFD) medium were found to differentiate into myofibroblasts in greater proportions when compared with dermal fibroblasts cultured in normal growth medium (NGM). However, addition of insulin to these cultures in GFD medium inhibited this increase in myofibroblast numbers in a dose dependent manner up to an 80% reduction (Mackie, 2004). This effect is repeatable in vivo in the murine model where insulin administered to excisional and incisional wounds was associated with a statistically significant decrease in α -SMA expression as detected by Western blotting (Mackie, 2004). The most effective dosing regime in this scenario was a once only dose (0.15 IU to 1.5 cm linear wound) of medium-length acting insulin (specifically Insulatard[®]) at the time of wounding and further applications of insulin worsened scarring (Mackie, 2004). Furthermore insulin did not adversely affect other important factors in wound healing, namely: speed of healing or tensile strength of healed wound (Mackie 2004).

Interestingly when other polypeptides that share some of the myriad other properties of insulin were also assessed for scar inhibiting behaviour, this showed that insulin-like growth factor (IGF) I and II are ineffective in this respect, suggesting insulin acts via its own receptor but the exact nature of insulin's effect on myofibroblast differentiation is unknown. Furthermore, insulin's efficacy in reducing myofibroblast numbers is inhibited in the presence of one or more of its physiological antagonists (adrenaline, noradrenaline, dexamethasone, and glucagon); however these antagonists do not significantly increase myofibroblast numbers within culture media (Mackie 2004).

1.11 Aim

The aim of this thesis is to determine the mechanism of action of insulin's effect on myofibroblast differentiation: whether it be direct on the mechanics of differentiation or whether it be on extrinsic factors that are required for differentiation. Knowledge of this kind may help us hone this ability by designing more effective drugs or treatment regimes. The second aim is to perform small-scale phase II clinical trial in an attempt to establish a good model in which to examine insulin's potential anti-scarring properties, to establish the feasibility of the study design and to establish safety and perhaps efficacy of treatment.

Chapter 2

Materials and Methods

2.1 Introduction

The solvents used in this study were analytical (AnalaR) grade supplied by BDH.

Tissue culture materials were supplied by Gibco and Sigma, the plastics by Greiner and Fisher and all the chemicals were supplied, at the best available grade, by Sigma, BDH and Fisher.

If alternative suppliers were used, this will be noted within the text.

All cell culture work was performed in a sterile class II laminar airflow hoods, (Laminar HB2448, Heraeus Instruments, Hanau, Germany) and cells were incubated in a Galaxy S incubator (Wolf Laboratories) at 37°C in a humidified atmosphere with 5% CO₂ concentration.

Fibroblast cell lines were established from skin and scar samples from patients that had scar revisions or related procedures. Local ethics committee approval was obtained (ethical approval number EC2002-20) and written consent was taken from every patient.

2.2 Primary Human Tissue Culture

Before experimentation, fibroblasts were routinely cultured and expanded for experimentation in normal growth media: Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal Calf Serum (FCS), 7mM 1M HEPES (pH 8), 1 unit/ml Penicillin/Streptomycin and 2mM L-Glutamine. This is referred to in the text as 10% NGM. All culture media were refrigerated at 4°C and used within 4 weeks of adding supplements. Media was warmed to 37°C prior to use.

2.2.1 Isolation and propagation of cells

The skin samples were prepared by removing epidermis and adipose tissue with sterile forceps and scalpel. These were cut into approximately 1mm² squares of dermis and placed in 25cm² tissue culture flasks and allowed to air-dry briefly to promote tissue adhesion to the plastic. 5mls of 10% NGM was then added. The cells were incubated at 37°C and the media changed twice weekly.

When the proliferating fibroblasts reached 80 % confluence (i.e. covering 80% of the surface area of the flasks), which usually occurred after approximately 3 weeks, the dermal tissue and the media were removed. The flasks were then washed twice with 4mls of versene (37°C) and then 1ml of 1:10 trypsin/versene mix (giving a final trypsin concentration of 0.25%) was added in order to detach the fibroblasts from the flask surface. Once the cells had rounded up the trypsin mix was neutralised by adding 4mls of 10% NGM to each flask. The resulting cell suspension was centrifuged at 1000g for

5 minutes to pellet the cells. Each pellet was re-suspended in 10%NGM and then sub-cultured in three T175 flasks.

The media was subsequently changed twice weekly until confluence was again achieved and the trypsinisation process repeated. Each flask culture was routinely split 1:3 until the passage number reached 8 after which the cells were no longer used for experimentation.

It is worth highlighting here that the insulin used in this previous work and in most of the experiments detailed in this thesis (except where specifically indicated) was laboratory grade bovine insulin. Bovine insulin has been chosen for use in the majority of experiments because of its lower expense and also for consistency across experiments.

Furthermore, the efficacy of the same source of insulin will naturally reduce over time as the protein gradually denatures. The bovine insulin preparations were renewed regularly to reduce the effects of protein denaturation that inevitably occurs over time.

2.2.2 Determination of cell number

Cells were counted using haemocytometer examined under an inverted phase contrast microscope (Olympus CK2, Olympus Optical Co., Japan).

2.2.3 Cryogenic storage

Stocks of cultured fibroblasts were cryogenically stored. To achieve this, the cells were trypsinised, pelleted and re-suspended in “freezing medium” (9mls FCS, 1ml DMSO).

Each T75 flask containing just semi-confluent cells was re-suspended in 3mls of freezing medium and then divided between three 1ml cryovials. The cryovials were insulated and placed into a -80°C for 24 hours before transfer to liquid nitrogen.

Retrieval of cells from cryostorage required rapid thawing in a 37°C water bath and gradually adding 9mls of 10% NGM to the suspension before centrifugation at 1000g for 5 minutes. The resulting pellet was then re-suspended in 10mls of 10% NGM and transferred to a T75 flask for further culture.

2.3 Crystal violet proliferation assay method

This is a dye uptake method, where the amount of dye and binding is directly proportional to the amount of DNA (Gillies et al, 1986). This is a rapid throughput assay that minimizes the cell numbers required and the amount of consumables used. This technique provides a good approximation of the cell numbers present and has been validated in this laboratory by comparison with direct cell counting for a number of fibroblast strains from different sources.

The method is performed as follows: Fibroblasts are seeded (5×10^3 cells per well in 200 μ l of test medium) in 96 well plates. At each time point the media is carefully aspirated and 100 μ l of staining solution added to each well. Following 10 minutes incubation at room temperature the solution is aspirated and the plate washed twice with PBS. 33% acetic acid is then added to solubilise the cells (100 μ l per well). Absorbance is measured in a plate reader at OD540nm with repeat measurements over the time course generating a plot of cell proliferation.

The crystal violet stain solution is made up as follows:

0.5% (w/v) of crystal violet

5% (v/v) of formol saline

50% (v/v) ethanol

0.85% (w/v) of NaCl

Sterile ddH₂O is added to adjust the final volume.

2.4 Immunohistochemistry

2.4.1 Fibroblast culture for immunohistochemistry

To enable analysis by immunohistochemistry fibroblasts were cultured on sterile 24 mm x 24 mm glass coverslips (Chance-Propper) in six well plates. In all experiments triplicate coverslips were set up for each experimental variable. The fibroblasts were harvested from tissue culture flasks as previously described to produce a cell suspension. The cells were counted using a Fuchs-Rosenthal haemocytometer and the number of cells per ml of medium calculated as previously described. The suspension was then centrifuged at 1000g for 5 minutes. The media was aspirated and then the pellet was then re-suspended in the desired volume of media, which varied depending on the need of each experiment. Two millilitres of media containing the desired number of cells was added to each well of the six well plates and these were then incubated overnight at 37°C to allow the cells to adhere to the coverslips.

The following day, designated as time zero (T0), the wells were washed with PBS (minus calcium and magnesium) and 2mls of test media added to the correct wells. The media was then changed at twice weekly intervals or more often if required by the experiment.

At each time point the coverslips were fixed in either methanol or formaldehyde depending on the requirements of the antibody. Media was aspirated from the plates and the wells washed with PBS. The coverslips remained in the methanol for at least 10

minutes before being allowed to air dry on tissue paper. Glass slides were labelled appropriately and when the coverslips were dry they were mounted onto the slides with DPX fixative, cell-side uppermost. The slides were stored at -20°C until required.

Alternatively, if formaldehyde fixation was required, the above process was repeated except room temperature 10% formaldehyde was used instead of methanol for at least 5 minutes. The slides were then stored as before at -20°C. Prior to immunostaining the formaldehyde fixed slides were treated with 0.2% Triton X-100 to make the tissue more permeable.

2.4.2 Immunohistochemistry technique

Various antibodies were used for immunohistochemistry in this research. The following method was used for all the antibodies, variations occurring only in the concentrations used. The methanol or formalin fixed coverslip cultures were ringed with a wax pen and rehydrated in PBS for 5 minutes. The primary antibody was diluted in PBS according to predetermined concentrations depending on the antibody (i.e. the lowest concentration at which staining was similar to maximal staining at high concentrations – see table 2.4.1 for concentrations) and applied in 100µl volumes to each coverslip. These were then incubated in a humidified tank for 1 hour at room temperature. The secondary antibody and counterstain was prepared: FITC (fluorescein isothiocyanate – a fluorophore) conjugated monoclonal antibodies (anti-mouse and anti-goat antibodies used depending on the primary, DAKO) and propidium iodide (nuclear counterstain, Sigma®) was diluted in PBS 1:400 and 1:40 respectively. A volume of 100 µl was again

applied to each coverslip. The slides were incubated in humidified conditions at room temperature in the dark (to preserve the fluorescence). After 1 hour's incubation the slides were washed again in PBS and then the coverslips were mounted with an aqueous mountant containing 14-Diazo dicyclo 2,2,2 octane (DABCO, Sigma[®]) to preserve the fluorescent signal. The slides were then analysed within an hour of mounting.

A Zeiss Axioskop microscope was used for examining the slides under UV light with a FITC filter. Propidium iodide-counterstained cell nuclei appeared red/orange and FITC labelled structures appeared green.

Each slide was assessed from at least three randomly selected fields and the images captured with a Leica DC 200 digital imaging system. The numbers of nuclei and secondary antibody-positive fibroblasts (e.g. myofibroblasts, in the case of α -SMA immunostaining) were counted and the relative proportions calculated.

Name	Antigen	Type	Concentration required for:		Manufacturer
			Immunohistochemistry	Western blotting	
Anti-human α -smooth muscle actin	Human α -smooth muscle actin	Mouse IgG	1:1000		Sigma [®]
Anti-mouse IgG FITC conjugate	Mouse IgG		1:400		Sigma [®]
Anti-human collagen type III	Human collagen type III	Mouse IgG		1:500	MP
Anti-EDA fibronectin antibody	Human EDA fibronectin	Mouse IgG	1:1000	1:1000	Chemicon [®] International
Anti-human integrin α V β 5	Human integrin α V β 5	Mouse IgG	1:1000	-	Chemicon [®] International
Anti-human MT-MMP1	Human membrane-type matrix metalloproteinase-1	Rabbit IgG		1:800	Chemicon [®] International
Anti-human LAP (TGF- β 1)	Human latency-associated peptide (TGF- β 1)	Goat IgG	1:1000	1:1000	R & D Systems
Anti-human TGF- β 1	Human TGF- β 1 and latent TGF- β 1	Mouse IgG	1:1000	1:1000	R & D Systems
Anti-human Thy-1	Human Thy-1	Mouse IgG	1:4000		Sigma [®]
Anti-human myosin	Human myosin	Mouse IgG	1:80	1:800	Sigma [®]
Anti-human tubulin	Human tubulin	Mouse IgG		1:1000	
Anti-human thrombospondin	Human thrombospondin	Rabbit IgG	1:1000		Biodesign Interna-

Anti-human FAK	Human FAK	Mouse IgG	1:1000	-	Chemicon [®] International
Anti-human phosphorylated FAK	Human phosphorylated FAK	Mouse IgG	1:800	-	Chemicon [®] International
Anti-human GAPDH	Human GAPDH	Mouse IgG		1:5000	
Anti-human IGF-II receptor	Human IGF-II receptor extracellular domain	Goat IgG	1:800	1:1000	R & D Systems
Anti-mouse IgG alkaline phosphatase conjugate	Anti-mouse IgG (whole molecule)	Rabbit IgG		1:1000	Sigma [®]
Anti-goat IgG alkaline phosphatase conjugate	Anti-goat IgG (whole molecule)	Rabbit IgG		1:1000	Sigma [®]
Anti-goat IgG peroxidase conjugate	Anti-goat IgG (whole molecule)	Rabbit IgG		1:1000	Sigma [®]

Table 2.4.1 Antibodies used in this thesis.

The flasks remained on ice for 20 minutes and were gently agitated three times. The fibroblasts were scraped from the floor of the flasks using separate cell scrapers for each flask and the cell lysates placed into 1.5ml centrifuge tubes.

The lysates were then treated with reducing or non-reducing buffer depending on the protein under investigation. All of the proteins examined in this thesis were treated with reducing buffer. 500µl of 2 x Laemmli reducing sample buffer (4% SDS, 120mM Tris pH6.8, 0.01% Bromophenol blue, 10% glycerol, 2% 1-mercaptoethanol) was added to

2.5 Protein detection methods

2.5.1 Protein sample preparation from monolayer cultured cells

For all of the western blotting experiments cells were cultured and collected from T75 flasks. First the media was aspirated from the flasks and then they were washed twice with ice cold PBS (without calcium or magnesium) and placed on ice. The PBS was drained and 1ml of SDS lysis buffer added to each flask.

The buffer consisted of:

0.1% sodium dodecyl sulphate (SDS)

125mM Tris pH 6.8

1mM Phenylmethylsulphonylfluoride (PMSF)

Sterile distilled water

The flasks remained on ice for 20 minutes and were gently agitated three times. The fibroblasts were scraped from the floor of the flasks using separate cell scrapers for each flask and the cell lysates placed into 1.5ml centrifuge tubes.

The lysates were then treated with reducing or non-reducing buffer depending on the protein under investigation. All of the proteins examined in this thesis were treated with reducing buffer. 500µl of 2 x Laemmli reducing sample buffer (4% SDS, 120mM Tris pH6.8, 0.01% Bromophenol blue, 10% glycerol, 2% 2-mercaptoethanol) was added to

each lysate. These were then heated to 97°C for 3 minutes, centrifuged at 1300xg for 3 minutes and then stored at -20°C. Prior to use the lysates were thawed at room temperature.

2.5.2 SDS Gel Formulation

Three different gel concentrations were used 10%, 15% (made in house) and 4%-20% gradient (supplied ready made by Invitrogen™) depending on the size of the protein being examined. Tables 2.5.1 and 2 detail the constituents.

	10% Separating gel	15% Separating gel
30% Acrylamide (Sigma)	5mls	7.5mls
1M Tris pH8.8	5.6mls	5.6mls
dH ₂ O	4.35mls	1.85mls
10% SDS	0.150mls	0.150mls
10% APS (Ammonium persulfate)	50µl	50µl
TEMED (Invitrogen)	10µl	10µl

Table 2.5.1 Constituents of SDS PAGE separating gels.

5% Stacking gel

30% Acrylamide (Sigma)	0.835mls
1M Tris pH6.8	0.625mls
dH ₂ O	3.515mls
10% SDS	0.050mls
10% APS	25µl
TEMED (Invitrogen)	5µl

Table 2.5.2 Constituents of SDS PAGE stacking gel.

The equipment used for SDS-PAGE electrophoresis was the Mini protean III kit (BioRad) that was set up according to the manufacturer's instructions. A 5% stacking gel, detailed above, was routinely used with all but the pre-made gels which came with an already prepared stacking gel. Once the gel had solidified the comb was removed and plate-gel combination carefully placed within the running tank and submerged in running buffer (10% x 10 stock, [0.025M Tris base, 0.192M glycine, 0.1% SDS in dH₂O] in 90% dH₂O). The first well was loaded with 15µl of precision plus pre-stained blue protein marker contains proteins of fixed molecular weights. The rest of the wells were loaded with the protein samples. Two sizes of wells were used and loaded with lysates mixed with equal volumes of 2 x Laemmli sample buffer to make up volumes of 20µl or 40µl, depending on the well size. The gels were run at 200V for 60 minutes or until the dye had reached the bottom of the gel.

2.5.3 Western blotting

The semi-dry blotting apparatus was prepared as per the manufacturer's instructions. Six pieces of filter-paper and a nitrocellulose membrane were all soaked in transfer buffer [10% x 10 stock, (25mM Tris base, 192mM Glycine in dH₂O) 20% methanol, 70% dH₂O]. Three pieces of soaked filter paper were placed on the base (anode) of the blotter, followed by the nitrocellulose membrane, then the gel and then the last three pieces of filter paper. Air-bubbles were carefully removed to avoid interference with protein migration.

The lid (cathode) was attached and the protein transferred at 25 volts for 45 minutes. The nitrocellulose membrane was then immersed in blocking buffer [5% BSA, 0.1% Tween-20 in 1 x TBS (10mM TRIS-base, 13.8mM NaCl, 2.7mM KCl)] on an agitator for 2 hours.

During electrophoresis the western blotter was prepared. Six pieces of filter-paper and a nitrocellulose membrane were all soaked in transfer buffer [10% x 10 stock, (25mM Tris base, 192mM Glycine in dH₂O) 20% methanol, 70% dH₂O]. Three pieces of soaked filter paper were placed on the base (anode) of the blotter, followed by the nitrocellulose membrane, then the gel and then the last three pieces of filter paper. Air-bubbles were carefully removed to avoid uneven protein migration. The lid (cathode) was attached and the protein transferred at 25 volts for 45 minutes. The nitrocellulose membrane was then immersed in blocking buffer on an agitator for 2 hours.

2.5.4 Antibody detection of proteins

After protein transfer the nitrocellulose membrane was incubated in blocking buffer for 2 hours on a rotary shaker at room temperature. This reduces the non-specific binding of antibodies. The membrane was washed three times in TTBS (1 x TBS + 0.05% Tween-20) wash buffer for 15 minutes and incubated overnight at room temperature with a solution of the primary antibody. The membrane was then washed a further three times in TTBS wash buffer for 15 minutes, and incubated with the secondary antibody in blocking buffer at room temperature on a rotary shaker for 1 hour. Two types of secondary antibody were used: those conjugated to alkaline phosphatase and those conjugated to horseradish peroxidase, depending on the sensitivity required.

2.5.5 Visualization of the alkaline phosphatase conjugate

After thorough washing of the membrane the protein bands were visualised using an alkaline phosphatase III substrate kit (Vector Laboratories, SK5300), according to the manufacturer's instructions. The position of the bands relative to the protein marker was checked to ensure they corresponded to the correct molecular weights of the proteins in question.

2.5.6 Image analysis

Membrane analysis was performed using the Lab Works Image Acquisition and Analysis Software System (UVP Laboratory Products). The membranes were scanned within an Epi Chemi II Darkroom using a digital camera. Bands were identified using the software above and Total Raw Density (TRD) of each band measured. The background TRD was also measured and subtracted from the value for each band. The TRDs of the bands of the protein were compared with the control (constitutively expressed, e.g. GAPDH) proteins in the same lanes to give relative band intensities. Results therefore represent the levels of the proteins of interest as a ratio to that of the control proteins.

2.5.7 Chemiluminescence (horseradish peroxidase) method

When only low levels of the protein of interest were present in the samples, horseradish peroxidase (HRP) conjugated secondary antibodies were used. The HRP enzyme can be used to catalyse a chemiluminescent reaction between two substrates (SuperSignal[®] West Pico Chemiluminescent Substrate) allowing a highly sensitive detection of the bands using photographic paper.

For the HRP method, incubation with the secondary antibody is followed by washing the membrane in TTBS for 20 to 30 minutes in order to reduce the background luminescence. The working solution is prepared by mixing equal volumes of the stable

peroxide solution and the luminal/enhancer solution to make 5mls per membrane. The blot is incubated with the working solution for 5 minutes. The blot is then placed in a membrane protector and taken to the dark room. The first film is placed on the membrane and left for 1 minute. Subsequent films, if needed, are placed on the membrane for 5 minutes, 15 minutes and 30 minutes.

The films were developed in standard developing solutions and fixative and then quantified with densitometric analysis of the digital images of the developed films (as per section 2.5.6).

2.5.7 Stripping membranes for reprobing

Stripping removes the primary and secondary antibodies so that the probing procedure can be repeated with different antibodies. This allows comparison between several different proteins, for example the protein of interest and a constitutively expressed loading control such as GAPDH.

A medium strength stripping buffer was used:

15g glycine

1g SDS

10ml Tween20

in 1L ultrapure water at pH 2.2

After the first analysis of a membrane using primary and secondary antibodies was complete, the membrane was incubated twice for 10 minutes in the stripping buffer. It was then incubated in two lots of PBS for 10 minutes followed by two washes for 5 minutes in TBST. The membrane was then ready for the blocking stage again, before staining with another primary antibody.

2.6 TGF- β Assay: Mink Lung Epithelial Cell (MLEC) Luciferase

assay (according to: Mazzieri et al., 2000)

This quantitative bioassay is based on the ability of TGF- β to up-regulate PAI-1 (plasminogen activator inhibitor-1) (Thalacker and Nilsen-Hamilton, 1992). TGF- β activity is determined using MLE cells permanently transfected with the expression construct p800neoLUC containing a truncated PAI-1 promoter fused to the firefly Luciferase reporter gene (Abe et al., 1994). The specificity and sensitivity of this assay are the result of using truncated PAI-1 promoter which retains the two regions responsible for maximal response to TGF- β (Keeton et al., 1991).

2.6.1 Materials

1. MLEC stably transfected with the expression construct p800neoLUC.
2. Geneticin stock – 250mg/ml in PBS – filter sterilised and stored aliquoted (5 μ l) at -20°C.
3. Luc-Screen system (supplied by Applied Biosystems) - a Luciferase detection kit.
4. Control medium and test (cell-conditioned) medium.
5. Recombinant TGF- β stock to make up standard curve.
6. Luminometer.

2.6.2 Methods

Routine culture of MLECp800neoLUC

Cells were grown in NGM plus supplemental geneticin (250 μ g/ml) as the selectant.

The cell cultures were split 1:50 approximately twice weekly.

Production of conditioned test media

Normal scar fibroblasts were plated at just sub-confluence in the test media (DFCS with and without supplemental insulin) for 4 hrs at 37°C and allowed to adhere to the flasks.

The cells were then washed twice with PBS, serum-free medium was added and then incubated at 37°C for 24 hours. The medium was then harvested and centrifuged to remove debris. Aliquots were stored at -80°C.

Heat-activation of TGF- β in conditioned media

The conditioned media was incubated for 10 minutes at 80°C and allowed to cool down before immediate use.

Standard Luciferase assay

The MLEC cultures were trypsinised and suspended at 5x10⁵ cells/ml in complete growth medium. 50 μ l (2.5x10⁴ cells) was added per well on a 96-well plate. The cells were then incubated for 3 to 4 hours to allow the cells to adhere to the plate. According to the experimental design detailed in section 5.5 the medium was replaced with 50 μ l of each of the following in triplicate:

- a. Control medium to determine the basal levels of TGF- β produced by the transfected MLEC.
- b. Control medium containing rTGF- β standard range; this assay can be used to measure TGF β in the 0.2-30pM range
- c. Conditioned medium from the test cultures to measure active TGF- β .
- d. Heat-activated conditioned media to measure total (latent plus active) TGF β .

The MLEC were then incubated for 16-20hrs at 37°C (no more than 20 hours in order avoid complications as a result of the effect of TGF- β on MLEC proliferation). The medium was then removed and replaced with 100 μ l/well of PBS (plus calcium and magnesium).

The Luc-Screen System was used to measure the Luciferase activity. Buffers 1 and 2 were warmed room temperature. Buffer 1 (50 μ l/well) was added to cells in 100 μ l of culture medium or PBS. Buffer 2 was then added and the plate was incubated for 10 minutes at room temperature. The temperature during the assay and measurement was between 23 and 27°C. The microplate was placed in the Luminometer and measured for 0.1-1sec/well.

2.7 Quantification of TGF- β secretion (enzyme linked immunosorbent assay)

TGF β 1 secretion was evaluated using a sandwich enzyme linked immunosorbent assay (ELISA) based on a modification of the procedure reported by Danielpour et al (1989).

Preparation of plates

96-well microtitre plates (Maxisorb, Nunc) were coated with monoclonal antibody to human TGF- β (R&D Systems) at a concentration of 2.5 μ g/ml in PBS. The plates were incubated for 2 hrs at room temperature (RT) followed by 16hrs at 4°C. The plates were washed twice with Tris buffered saline, pH 7.3 containing 0.05% Tween-20 (TBST). Non-specific binding sites were blocked by incubation with PBS containing 5% Tween-20, 5% sucrose, 1.4% bovine serum albumin for 1 hr at RT followed by four washes with TBST.

Preparation of media

Fibroblast conditioned media was obtained from cells that had been pre-incubated in the different test media for 14 days, washed well and then incubated in serum-free medium for 24 hrs. This conditioned medium was collected and diluted in TBST supplemented with 1.5% bovine serum albumin diluent where necessary, acidified with 24 μ l/ml of 5M HCl for 15 min at RT then neutralised with 40 μ l/ml of 1M HEPES pH 8/5M Na OH (5:3 ratio). The plates used to condition the media were then trypsinised and the cells counted to allow normalisation of the TGF- β concentrations to be per 10⁵

cells. The acidified/ neutralised samples (100µl/well) were then incubated on plates for 1 hr at RT.

Quantification method

After washing, the plates were incubated with biotinylated chicken anti-human TGF-β (R&D Systems) at a concentration of 50ng/ml for 1hr at RT. After six washes with TBST, the plates were incubated with ExtraAvidin- alkaline phosphatase for 1 hr and subsequently washed. Absorbance was measured at 450nm using a microplate spectrophotometer (BioRad Model 550) following addition of the substrate OPD 1mg/ml for 1 hr at RT. Serial dilutions (3.9-2000pg/ml) of recombinant human TGFβ1 (R&D Systems) were used to prepare a standard curve and the experimental results were expressed as pg per 1×10^5 cells.

2.8 RT-PCR Methods

2.8.1 RNA extraction

Total RNA was extracted from cells using the TRIZOL[®] reagent (Invitrogen[™]) as follows:

Flasks or wells containing the cells of interest were washed in cold PBS (4°C) and TRIZOL[®] was added (0.5mls/well of a 6 well plate or 1.5mls to a T75 flask). After five minutes incubation at room temperature the cells were removed from the surface with a cell scraper and the cell preparation placed into a sterile 1.5ml centrifuge tube.

Chloroform was then added (200µl per ml TRIZOL[®]) in a fume cupboard. The tubes were shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes.

The extracts were then centrifuged at 8-11,000g at 4°C for 15 minutes which separates the extracts into an organic phase at the bottom and an upper aqueous phase containing the RNA at the top. This phase was transferred to a fresh tube and 0.5mls of 80% isopropanol added. The tubes are then shaken for 15 seconds and incubated at room temperature for 10 minutes. The tubes are then centrifuged again at 11,000 g for 30 minutes at 4°C to pellet the RNA. The supernatant was removed and 1ml of 75% DEPC-ethanol added to each tube followed by mild vortexing for 10 seconds to wash the pellet. The RNA was re-pelleted by centrifuging at 6,000 g for 5 minutes. The supernatant was then carefully removed and the pellet allowed to air-dry for 10 minutes. Finally the RNA is dissolved in 40 µl DEPC-water by gentle pipetting. The samples were stored at -20°C until use.

2.8.2 Determination of RNA yield and quality

The concentration and purity of RNA were determined prior to generating cDNA. To do this, 1µl of extracted RNA sample was diluted in 999µl of DEPC treated water and the absorbance determined with a spectrophotometer (ComSpec M330). The machine was zeroed using 1000µl of DEPC water only. Absorbance readings were taken at 260nm (A_{260}) and at 280 nm (A_{280}).

Purity was determined by calculating the ratio of the A_{260} : A_{280} absorbancies.

Absolutely pure RNA samples give a ratio of 2:0, but ratios between 1.7 and 2.1 are acceptable. (Sambrook et al 1989)

The formulae for these calculations are as follows (Sambrook et al 1989):

RNA concentration ($\mu\text{g}/\mu\text{l}$) = [$A_{260} \times 40 \times \text{dilution factor (1000)}$]/1000 (μl)

RNA yield (μg) = RNA concentration \times total volume of pooled RNA

2.8.3 cDNA preparation

RNA from the stock solutions was diluted in fresh sterile PCR grade microfuge tubes to give a concentration of 5µg RNA in 8µl DEPC-water. The samples were heated to 65°C in a heating block for 10 minutes and then put on ice for 5 minutes.

The reagents for the working mix were thawed on ice with the exception of reverse transcriptase which was kept at -20°C until immediately before use. The RT working mix was prepared in a fresh sterile 1.5ml centrifuge tube (4µl 5× RT buffer, 2µl 0.1M DTT, 1µl oligo-dT primer, 1µl DEPC-water, 2µl 10mM dNTP, per RNA sample). 10 µl of RT working mix was added to each sample along with 1µl of RNA guard (and RNase inhibitor) and 1µl of RT (200IU/ml). The samples were then incubated for 1 hour on a heating block at 37°C until the reaction was terminated by heating to 75°C for 10 minutes. The resulting cDNA samples were then centrifuged at 13000g for 1 minute and stored at -80°C until they were used.

2.8.4 Polymerase chain reaction (PCR)

PCR allows the amplification of specific sequences of DNA from very small samples. PCR requires two primers (short single stranded DNA) that are complementary to the two ends of the sequence of interest in a solution containing DNA polymerase and nucleotides. PCR is a cyclical process in which the DNA is denatured to become single stranded, the primers are allowed to anneal with the single strands and then they are extended by DNA utilising nucleotides. A single strand of DNA is synthesised with a sequence complementary to the template strand. Thus with each cycle there is exponential amplification of the chosen DNA sequence.

2.8.5 Oligonucleotide primers

Specific oligonucleotide primers were chosen for the amplification of several genes (see table 2.8.1). The primers used spanned two intron-coding regions and one exon

region. Primers were checked for specificity and cross-reactivity using BLAST. The housekeeping gene GAPDH was primarily used as the internal cellular control although other housekeeping genes were used too. All the primers used were synthetic primers and purchased from MWG Biotech. The base sequences of these primers are shown in table 2.8.2.

Primer	Manufacturer
GAPDH	MWG
TGF- β 1	MWG
TGF- β 2	MWG
TGF- β 3	MWG
IGF-II receptor	MWG
Tubulin	MWG
Myosin	MWG

Table 2.8.1 The RNA primers used in this research.

2.8.6 The PCR reaction

The reagents were stored on ice with the exception of DNA polymerase which was kept at -20°C until use. DEPC-water was used as negative control for detection of contamination of stock solutions. 2 μ l of each DNA sample was aliquotted into fresh 0.5ml PCR grade microfuge tubes. In sterile 1.5ml centrifuge tube the PCR working mix was prepared (2 μ l 10 x PCR buffer, 2 μ l 2mM dNTPs, 2 μ l of each forward and reverse housekeeping primers (10pM GAPDH), 2 μ l of each forward and reverse test primers (10pM), 1 μ l DMSO, 4.75 μ l DEPC-water and 0.25 μ l 5U/ml 2 μ l DNA polymerase). 18 μ l of working mix was added to each aliquot of cDNA and mixed. 2 drops of mineral oil overlaid the mix to reduce evaporation. Various PCR programs

were used depending on the gene of interest. PCR reactions occurred in the Techgene-Techne, Jenkons PLS PCR machine.

2.8.7 PCR gels

2% agarose gels were made by dissolving 2g agarose in 100mls 1 x TAE buffer (for 1L: Tris-base 0.04M, EDTA- Na_2 -salt 0.001M, acetic acid 0.2 M). The liquid was heated in microwave for 3 minutes and 5 μl ethidium bromide added for fluorescence. The liquid was then poured into a mould with a comb and allowed to set over 30 minutes.

The solid gel was placed in an electrophoresis tank and submerged in 1 x TAE buffer. 20 μl of each PCR sample was mixed with 2 μl of blue dye (15% Ficoll 400 in dH_2O , 0.25% xylene cyanol FF, 0.25% Bromophenol blue, 30% glycerol in dH_2O). The samples were loaded into the gel alongside a well containing 6 μl of 1,2,3 DNA ladder (Invitrogen-Cat 15613029). The gel was run at 100V for 25 minutes or until the dye front had migrated down to two thirds of the gel.

The gel was then examined and photographed using the UVP camera-computer system and analysed using Lab Works version 4.0 (UVP, Bioimaging Systems).

2.8.8 Image analysis

Gel analysis was performed using the Lab Works Image Acquisition and Analysis Software System (UVP Laboratory Products). The gels were scanned within an Epi

Chemi II Darkroom using an ultraviolet source and a digital camera. The intensity of each band was quantified to give total raw densities (TRD). The background TRD was also measured and subtracted from the value for each band. The TRDs of the bands of the protein were compared with the constitutively expressed genes such as GAPDH to give relative band intensities. Results therefore represent the levels of mRNA expression of the genes of interest as a ratio to that of the constitutively expressed genes.

2.8.9 Real Time Reverse Transcriptase PCR

Two μ l of the first strand cDNA product was used for amplification in triplicate in a 25 μ l reaction solution containing 12.5 μ l of SYBR Green PCR Master Mix (StratageneTM) and 10pM of each primer as per manufacturer's instructions. The PCR reaction was performed on a MX3000P (StratageneTM, La Jolla, California). The PCR program consisted of an initial denaturation where the reaction was incubated for 10 minutes at 95°C, in the second step the DNA was amplified for 55 cycles of 30 seconds at 95°C annealing primers for 1 minute at 60°C and an extension at 72°C for 30 seconds. Dissociation curve was performed to ensure no primer-dimers were present at the end of each PCR run. Normalisation was with two housekeeping genes – ACTB and GAPDH.

Gene	Sequence
TGF- β 1	F5' GCT CCA CGG AGA AGA ACT GCT3' R5' CTG CTC CAC CTT GGG CTT GC 3'
TGF- β 2	F5' CTT TGG ATG CGG CCT ATT GCT 3' R5' AGC TGT TCA ATC TTG GGT GTT T3'
TGF- β 3	F5' AGA AGA GGG TGG AAG CCA TT 3' R5' ATT GGG CTG AAA GGT GTG AC 3'
ACTB	F5' CAT CGA GCA CGG CAT CGT CA 3' R5' TAG CAC AGC CTG GAT AGC AAC 3'
GAPDH	F5' CCC CTT CAT TGA CCT CAA CTA 3' R5' GCC AGT GGA CTC CAC CGA CG 3'
IGF-IIR	F5' CAA CAT CTG TGG AAG TGT GG 3' R5' GGT CTC GTA GTG TGT CTA TG 3'

Table 2.8.2 Base sequences of the primers used in this thesis.

2.9 Statistical Analysis of Data

All of the data in this thesis was analysed using SigmaStat (Jandel) software. The tests used were paired Student's t-tests. When other tests were used this is stated in the text. Statistical significance was assumed when $p < 0.05$.

Chapter 3

Examining the mechanism of insulin's inhibition of myofibroblast differentiation

3.1 Introduction

The discovery by Linge et al. (2004) that serum contains a substance, now known to be insulin that inhibits the differentiation of fibroblasts into myofibroblasts is the rationale for undertaking this thesis. This chapter seeks to initially reproduce this finding in order to then begin investigating its mechanism of action.

The first section explores whether the author can not only generate the same raised myofibroblast numbers in growth factor deactivated media as Linge et al (2004) but in addition whether this phenomenon can be inhibited with the addition of insulin, as had previously been achieved by Linge et al (2004). This chapter introduces the various media that will be used in the research and an understanding of their varying properties is crucial in interpreting the results. In the original studies performed by Linge et al. (2004) in attempting to produce a more defined medium that supported cell survival, it was discovered that fetal calf serum contains an inhibitor of myofibroblast differentiation. This medium was Dulbecco's Modified Eagle's Medium (DMEM) containing dithiothreitol (DTT)-treated fetal calf serum FCS at a concentration of 4% as the treated serum is toxic at higher concentrations. This medium is referred to as DTTM from hereon. The purpose of this treatment was to produce a medium devoid of active growth factors (particularly TGF- β 1 but also incidentally insulin). The three-dimensional structure of many polypeptides is intrinsic to their activity and is held together in many cases by disulphide bonds. Dithiothreitol catalyses the breaking of these disulphide bonds and thus deactivates almost all growth factors. This media was

required so the effects on myofibroblast differentiation of specifically added growth factors could be assessed in a more defined environment. Unexpectedly however, it was observed that myofibroblast numbers were actually increased in the DTTM compared to in NGM and this crucial experiment will be undertaken in this chapter to demonstrate its reproducibility in the author's hands. The implication of this finding is that FCS contains an inhibitor of myofibroblast differentiation. This was found to be insulin and its addition to DTTM could prevent the increase in myofibroblast numbers with an EC₅₀ of approximately 20-40 nM/ml whereas the related growth factors IGF-I and IGF-II did not exhibit this effect when evaluated over a wide range of concentrations (Linge et al. 2004). To consolidate this original finding 4% DTTM will be used in this thesis but this time with normal scar fibroblasts rather than non-scar dermal fibroblasts. However, insulin amongst other growth factors can also be removed from FCS without DTT treatment (and its attendant toxicity) by simply dialysing the FCS with molecular weight cut-off of 14kDa (see table 3.1.1). Mackie (2004) tested this medium and found 9.4% myofibroblasts in dermal (note not normal scar) fibroblast cultures after 14 days in medium containing 10% dialysed serum (DFCS). This contrasted with 2.3% myofibroblasts in NGM (containing 10% untreated serum) and a dose response drop for myofibroblasts of 6.4% to 1.7% myofibroblasts in DFCS plus bovine insulin at concentrations from 12.5 to 200 µg/ml respectively. These results demonstrated the same statistically significant changes as that seen in DTTM. In this thesis both DTTM and DFCS media will be used to provide continuity and consolidation of the original work. A concentration of 4% will be used for DTTM as in the original work and also for DFCS to minimise any proliferative effects of higher

concentrations. However, the absence of DTT in DFCS could make it a more favourable culture medium for fibroblast proliferation than DTTM. This could potentially confound results by permitting an increase in fibroblast cell numbers which might affect the percentage of myofibroblasts detected thus giving a misleading appearance of the level of myofibroblast differentiation. Furthermore, insulin is an anabolic hormone with a multitude of effects on cell metabolism and therefore might also influence fibroblast proliferation even in these growth factor reduced media. Therefore, this chapter will also compare fibroblast proliferation in the different test media with and without added insulin.

Growth Factor	Major role in wound healing
EGF	Mitogenic for most epithelial tissues, fibroblasts, endothelial cells
Some TGF α	Potent angiogenic factor
IGF-I	Mitogenic for fibroblasts, bone cells, neural tissues, haematopoietic cells, endothelial cells
IGF-II	Mitogenic for fibroblasts
Insulin	Mitogenic for many cell types. Major hormone of glucose homeostasis

Table 3.1.1 Table showing some of the major growth factors thought to have been removed during preparation of dialysed FCS and that are therefore present in Normal Growth Medium. DFCS is prepared by dialysing 100% FCS at 4°C over five days with

multiple exchanges of excess PBS using a membrane permeable to molecules less than 12-14kDa in size, including insulin.

There are essentially two ways in which insulin may exert its myofibroblast inhibiting actions: firstly, by modifying the signals (growth factors) or the detection of those signals (receptors) that induce fibroblast differentiation or secondly, by changing the response to those signals that is by preventing the final step – differentiation. Much of this thesis and much of scarring research generally is aimed at the first mechanism. It would be a straight forward mechanism of action if insulin does prevent the action of a growth factor such as TGF- β 1 and this possibility will be examined in this chapter. In contrast, a mechanism involving the differentiation process itself is more complex being intracellular, multi-staged, far less understood and involving the activation, inactivation and interaction of various genes, and their gene products. Thus designing an anti-scarring agent that works at the level of the differentiation process is potentially a greater challenge than the design of an agent that works by modifying the signals that induce differentiation in the first place. It is therefore of primary importance to establish if insulin-treated cells are capable of undergoing differentiation given the correct stimulus.

Insulin's possible mechanism of action can be further subdivided according to the different requirements for differentiation into myofibroblasts. The differentiation process is known to be induced by extracellular growth factors that in turn require the presence of other extra- and intracellular factors (Darby et al. 2007) (for more detailed

discussion see sections 1.4 – 1.5). So for example, for TGF- β 1 to exert its pro-myofibroblast effect both EDA-fibronectin and wound tension also need to be present (Beanes et al. 2003). At the other end of the differentiation pathway, other factors again need to be present for the fibroblast to take the final step of expressing α -SMA, such as stress fibres and mature focal adhesion complexes (Goffin et al. 2006). Thus insulin could inhibit the fibroblast-myofibroblast transition at either end of the differentiation process and this can be studied by examining insulin's effects on the expression of various substances involved in myofibroblast differentiation. Of course, there are multiple intervening steps and other important factors in the differentiation process that should also be examined but because of the limitations of time only the key factors will be investigated in this chapter. These are EDA-fibronectin, stress fibres, α V β 5 integrin, FAKinase and phosphorylated FAKinase, Thy-1, thrombospondin 1, MT1-MMP, collagen and the lipid expressing phenotype. Details of how each of these factors relates to myofibroblast differentiation is given before each relevant results section.

Aims

- To determine whether the results of Linge et al. can be reproduced in the author's hands, but using normal scar fibroblasts rather than dermal fibroblasts from intact skin.
- To establish the relative affects on fibroblast proliferation of the test growth media with and without addition of insulin.

- To demonstrate whether insulin treatment prevents the myofibroblast-inducing action of (exogenous) active TGF- β 1 on fibroblasts.
- To reveal whether insulin inhibits the expression of one or more of the important factors involved in the differentiation of the myofibroblast or its induction.
- To see whether insulin induces the differentiation of fibroblasts into a lipid expressing phenotype (at the expense of differentiation into myofibroblasts).

3.2 Comparison of the proportion of myofibroblasts in fibroblast cultures in growth factor deactivated media and normal growth medium

3.2.1 Introduction

This experiment aims to repeat the original findings by Linge et al (2004) of raised myofibroblast numbers in growth factor deactivated media compared with normal growth media. This is important for the correct interpretation of future experiments and also allows familiarity with the experimental techniques. The same growth factor deactivated media is used in this experiment as in the original work, namely DMEM with 4% dithiothreitol treated fetal calf serum (DTTM). This medium was used in the original work because it permits only a minimal proliferation of fibroblasts (thus making the interpretation of myofibroblast numbers easier) (Linge et al., 2004). However, human *normal scar* fibroblasts are used here rather than *dermal* fibroblasts. Normal scar fibroblasts will be used in the rest of the research as the fibroblasts in scars and wounds that differentiate into myofibroblasts and upon which insulin should act if it is to be a successful antiscarring therapy.

3.2.2 Method

Normal scar fibroblasts were grown on coverslips in 6-well plates for 17 days from a density at day 0 of 1×10^5 cells per well. The initial culture media was DTTM which is depleted of active polypeptides including insulin. The day after plating the time 0 coverslips were harvested and the media on the remaining wells changed to either 10%

NGM or fresh DTTM as appropriate. The coverslips were then fixed with methanol on days 3, 7, 10, 14 and 17 before immunostaining for α -SMA. Each coverslip was examined under x40 magnification in three random fields. The percentage of myofibroblasts was calculated. N=3 cell strains from normal scar tissue were used and each cell strain was repeated in triplicate.

3.2.3 Results

At day 3 and beyond there was a significant (T-test comparison: $P=0.009$, day 3; $P=0.002$ days 7 to 17) increase in the percentage of myofibroblasts in DTTM (range – 15 to 30%) compared to NGM (approximately 5%) that reached a maximum at approximately 14 days (see figs. 3.2.1a- & b and 3.2.2), thus reproducing the findings of Linge et al (2004). It was also noted that the microscopic appearance of myofibroblasts varied considerably in terms of the intensity of staining of the α -SMA fibres even within the same coverslip.

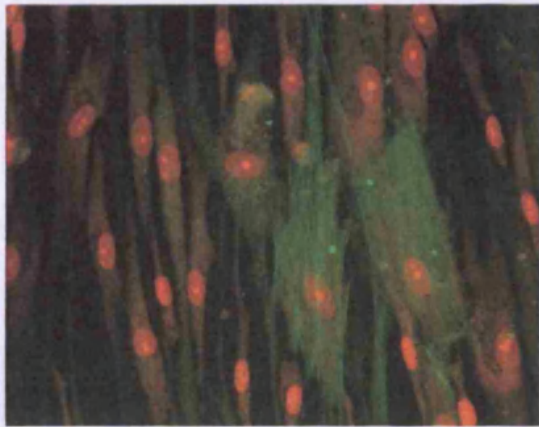


Fig. 3.2.1a Typical appearance of normal scar fibroblasts cultured in NGM for 14 days and stained for α -SMA (green) with propidium iodide (red) counterstain. This shows most cells displaying homogenous non-specific autofluorescence with only occasional cells displaying the fibrillar α -SMA-positive stress fibre structures. X40 mag, fluorescence microscope.

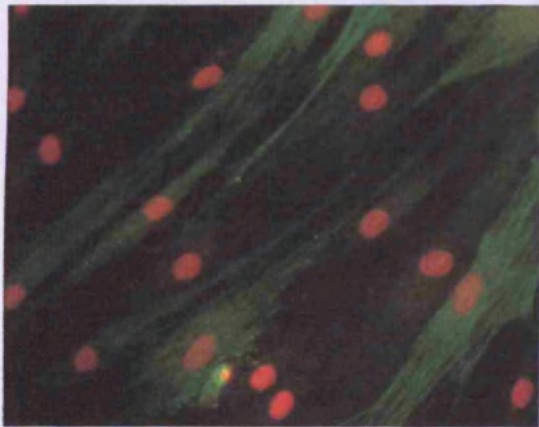


Fig. 3.2.1b Typical appearance of normal scar fibroblasts cultured in 4% DTTM for 14 days and stained for α -SMA with propidium iodide counterstain. X40 mag, fluorescence microscope. Note fewer nuclei presented in the field of view compared with fig. 3.2.1a but a higher proportion of myofibroblasts.

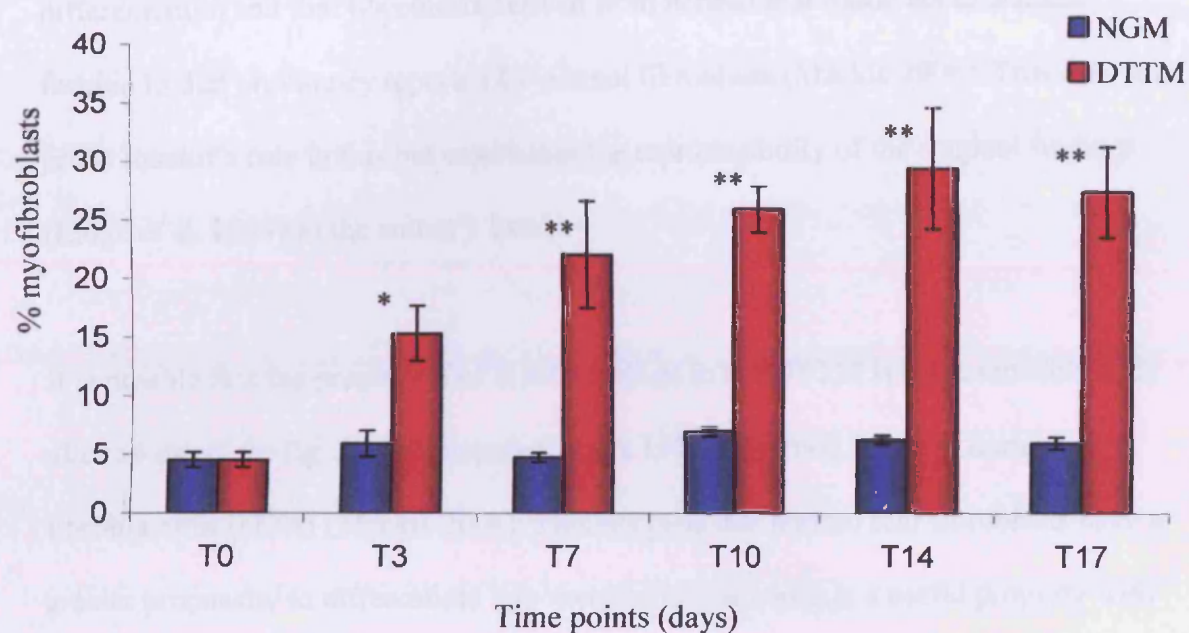


Fig. 3.2.2 Graph showing the mean proportion of myofibroblasts of three normal scar fibroblast strains, expressed as a percentage of the total number of cells, after 17 days treatment in NGM or DTTM. Error bars represent standard errors from the mean of three cell strains performed in triplicate. Student t-test * $P=0.009$, ** $P<0.002$.

3.2.4 Discussion

These findings support the hypothesis that DTT-treated serum induces myofibroblast differentiation and that fibroblasts derived from normal scar tissue act in a similar fashion to that previously reported for dermal fibroblasts (Mackie 2004). This does not prove insulin's role in this but establishes the reproducibility of the original findings (Linge et al. 2004) in the author's hands.

It is notable that the proportion of myofibroblasts in the DTTM is approximately 25% after 14 days (see fig. 3.2.2) compared with a 10% proportion found in dermal fibroblasts in DTTM (Mackie 2004). This suggests that normal scar fibroblasts have a greater propensity to differentiate into myofibroblasts which is a useful property with respect to future experiments in which the proportions of myofibroblasts between cultures will be compared. However, the proliferation rate in NGM may be naturally higher than in the growth factor reduced test media, DTTM and DFCS. This could make interpretation of results more difficult and so an investigation of fibroblast proliferation in different media will be undertaken in section 3.3.

3.3 Comparison of fibroblast proliferation in the test media

3.3.1 Introduction

Normal growth medium contains 10% FCS and is therefore rich in growth factors including insulin and TGF- β 1. Therefore when investigating the effects of adding exogenous insulin and TGF- β 1 to cell cultures NGM is a poor control medium. Another disadvantage of NGM is that it may promote cell proliferation more than the growth factor deactivated media which may complicate interpretation of the proportions of myofibroblasts in different fibroblast cultures. This is because these differences could be due to inhibition of myofibroblast differentiation or differences in the proliferation of fibroblasts (and myofibroblasts). Serum-free media would prevent proliferation completely but would also be detrimental to cell health and eventually cause apoptosis. Thus various test media have been described (see section 3.1) that allow maintenance of all cells over long periods of time (over 17 days) but that may have different proliferative effects. The purpose of this section is to see whether the main media used in this research demonstrate any significant differences in terms of fibroblast proliferation and additionally see whether insulin itself has a significant effect on proliferation. In addition, observation of cells during the initial experiments (see section 3.2) suggested there were differences in cell health (judged by microscopic appearance) and proliferation between the different media. In particular, DTTM seemed to be the least favourable medium with cells appearing sparser and polydendritic (see fig. 3.3.1a and b). A proliferation assay would also help to determine the significance of this impression.

Fig 3.3.1

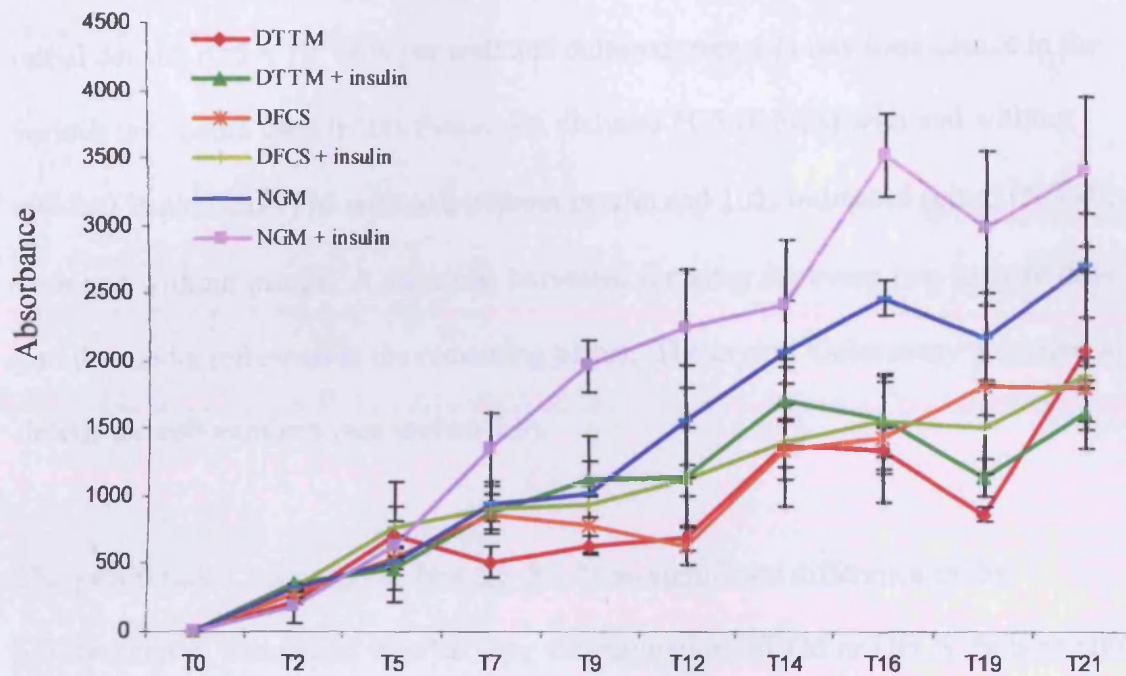


Figure 3.3.2 Crystal violet proliferation assay. Normal scar fibroblasts were cultured in 96 well plates over a 21 day time course in various test media: DFCS with and without insulin, DTTM with and without insulin ($5\mu\text{g/ml}$) and normal growth medium (NGM), with and without insulin. $N=4$. NGM + insulin shows significantly higher proliferation beyond day 9 (T-test $P<0.05$) compared to all the other media except NGM.

3.3.2 Results

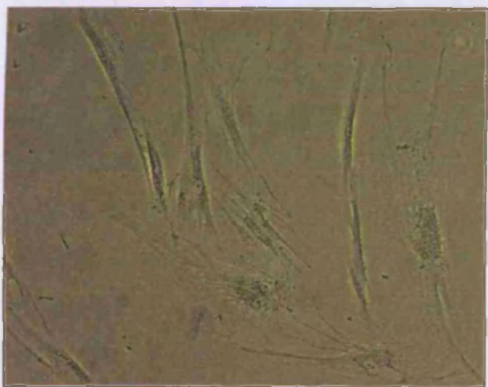
Four normal scar fibroblast cell strains were seeded in triplicate on 96 well plates at an initial density of 5×10^3 cells per well and cultured over a 21 day time course in the various test media used in this thesis: 4% dialysed FCS (DFCS) with and without insulin ($5\mu\text{g/ml}$), DTTM with and without insulin and 10% untreated serum (NGM), with and without insulin. A plate was harvested for assay for every two to three days and the media refreshed in the remaining plates. The crystal violet assay was used to determine cell numbers (see section 2.3).

The proliferation assay shows (see fig. 3.3.2) no significant difference in the proliferation of fibroblasts whether they are cultured in DTTM or DFCS. In both DFCS and DTTM addition of insulin does appear to give an overall marginal increase in proliferation but this does not reach significance and by day 21 the difference is minimal. The fibroblasts cultured in NGM plus insulin however, show significantly increased proliferation compared to all the other media except NGM. The addition of insulin to NGM produces a non-significant trend towards increased proliferation compared to NGM alone.

Fig 3.3.2



a) DFCS



b) DTTM

Fig. 3.3.1 Typical appearance of fibroblasts cultured for 14 days in a) DFCS and b) DTTM. Light microscopy x10. The cells cultured in DFCS tend to have a more homogenous and less dendritic appearance.

3.3.3 Discussion

These results (see fig. 3.3.2) demonstrate that there is no statistically significant change in the increase in cell number seen on culture in all of the different test media – NGM, DTTM and DFCS. Therefore although it is clear that all test media support the proliferation of normal scar cells, they do so equally. Also it is important to note that supplementation of both forms of growth factor depleted media (DTTM and DFCS) with insulin also does not significantly affect cell proliferation. The results suggest that any change in myofibroblast numbers between the different test media with and without additions of insulin is unlikely to be due to or complicated by an effect on cell proliferation.

3.4 Does insulin prevent the induction of myofibroblast differentiation by exogenous TGF- β 1?

3.4.1 Introduction

Section 3.2 demonstrates the increased numbers of myofibroblasts seen in growth factor deactivated media (DTTM) compared to NGM whilst section 3.3 suggests this is not simply due to differences in cell proliferation. Mackie (2004) repeated the work of Linge et al. (2004) to show that addition of bovine insulin and subsequently certain formulations of recombinant human insulin (Mackie, 2004) to growth factor deactivated medium over 14 days significantly inhibits the increase in myofibroblast numbers in a concentration dependent manner. However, these culture conditions are very different from the wound environment in several respects but perhaps most importantly with regards to myofibroblast differentiation they lack paracrine TGF- β (active TGF- β released by platelets and other cells at the time of wounding). As sections 1.4 and 1.5 discuss, TGF- β 1 is the most important inducer of the differentiation of fibroblasts into myofibroblasts and its inhibition with neutralising antibodies significantly inhibits scar formation (Shah et al., 1995). Thus for insulin to exert its antifibrotic action does it in some way ameliorate the effects of paracrine TGF- β 1? This question can be simply tested in vitro by adding exogenous active TGF- β 1 to cultures to mimic paracrine TGF- β 1. This is the purpose of this section.

3.4.2 Results

Normal scar fibroblasts were cultured on sterile coverslips in NGM alone or supplemented with 2ng/ml TGF- β 1 alone, 5 μ g/ml insulin alone, or insulin plus TGF- β 1 for 4 days. The coverslips were then harvested for fixation and immunohistochemical staining for α -smooth muscle actin. The proportion of cells staining positively was then calculated.

Figure 3.4.1 indicates that there is no inhibition of myofibroblast differentiation in NGM when insulin alone is added. Furthermore, when exogenous TGF- β 1 alone is added to NGM, the proportion of myofibroblasts significantly jumps from 10% to approximately 40%. However, if the medium is supplemented with insulin and TGF- β 1 this increase in myofibroblasts is not inhibited and again reaches 40%.

This experiment was then modified in two important respects to test if long-term treatment, that is for a period similar to that effective in the growth factor depleted test media experiments of the previous sections, could inhibit TGF- β mediated differentiation. Firstly, NGM was replaced by DFCS to give more controlled culture conditions and secondly the duration of insulin treatment was extended to 14 days prior to TGF- β 1 treatment. Normal scar fibroblasts were grown on sterile coverslips in either DFCS alone or with 5 μ g/ml insulin added for a period of 14 days (media refreshed twice weekly). On the 14th day the media was refreshed with the appropriate media alone or further supplemented with either 0.5 or 2ng/ml of TGF- β 1 and cultured for a further 4 days before fixation and staining of coverslips for α -smooth

muscle actin. The proportion of cells positive for α -smooth muscle actin was counted and the results normalised between different cell strains by calculating as a percentage of the proportion of myofibroblasts seen in DFCS media only.

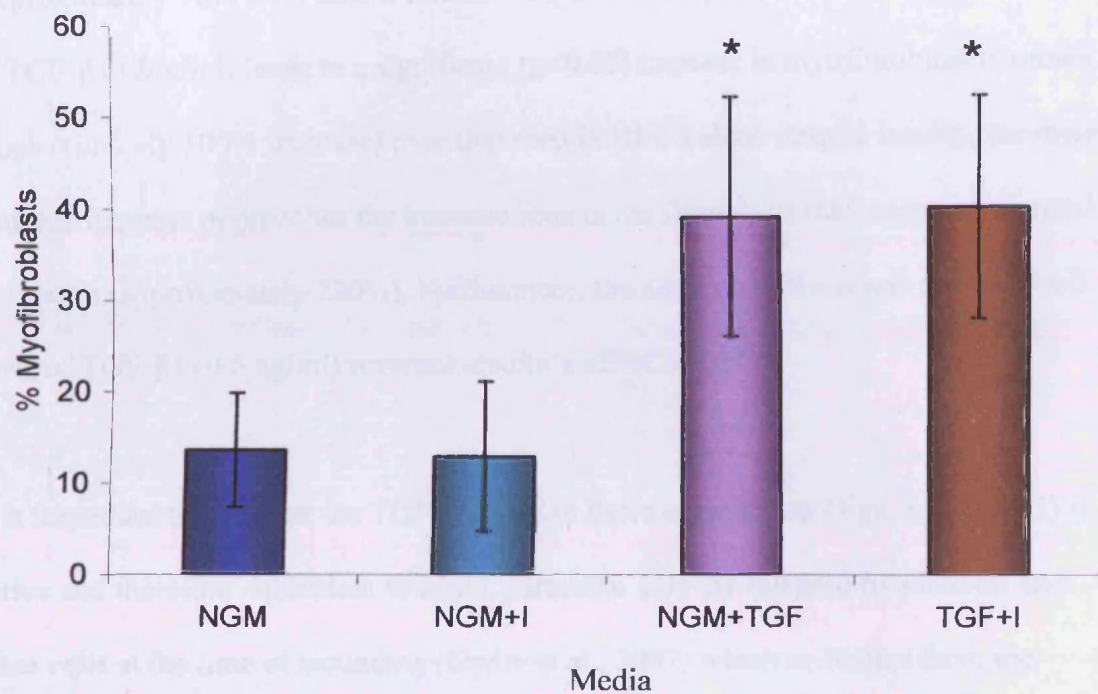


Figure 3.4.1 Does insulin interfere with TGF β 's ability to induce myofibroblast differentiation? Normal scar fibroblasts cultured in NGM alone or supplemented with 2ng/ml TGF- β 1 alone, 5 μ g/ml insulin alone, or insulin plus TGF- β 1 for 4 days. Percentage of α -SMA positively staining cells is shown. N = 4. Error bars represent the standard deviation. Student t-test: $p < 0.05$ (*).

The results following these modifications (fig. 3.4.2) show firstly, that long-term continuous insulin treatment for 14 days (over same time course as experiments in growth factor depleted test media) is associated with decreased myofibroblast numbers (approximately 70% less) after a further 4 days. Secondly, addition of the higher dose of TGF- β 1 (2ng/ml) leads to a significant ($p<0.05$) increase in myofibroblast numbers (approximately 100% increase) over that seen in DFCS alone despite insulin treatment and this increase approaches the increase seen in the fibroblasts that were not exposed to insulin (approximately 220%). Furthermore, the addition of the lower (suboptimal) dose of TGF- β 1 (0.5 ng/ml) reverses insulin's effect in DFCS.

It is important to note that the TGF- β added in these experiments (figs. 3.4.1 and 2) is active and therefore equivalent to active paracrine TGF- β 1 released by platelets and other cells at the time of wounding (Darby et al., 2007) which is distinct from the inactive autocrine TGF- β 1 that fibroblasts release in response to paracrine TGF- β 1.

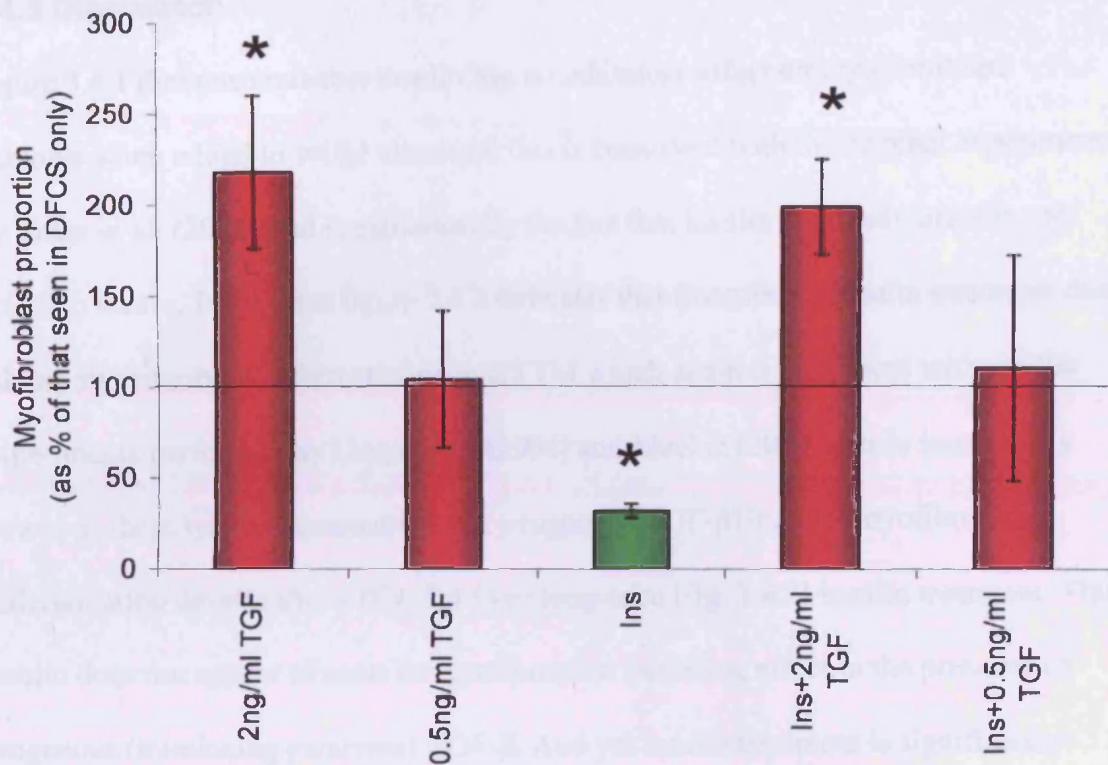


Figure 3.4.2. Normal scar fibroblasts cultured in DFCS alone and DFCS with 5 μ g/ml insulin added for 14 days. On the 14th day the media was refreshed with DFCS alone or further supplemented with either 0.5 or 2ng/ml of TGF β 1 and cultured for a further 4 days. of cells staining positive for α -smooth muscle actin is shown expressed as a percentage of the proportion in DFCS media only. Error bars = SEM. N = 3.* p<0.05 significant difference from DFCS only control.

3.4.3 Discussion

Figure 3.4.1 demonstrates that insulin has no inhibitory effect on myofibroblast numbers when added to NGM alone and this is consistent with the original experiments by Linge et al. (2004) and is explained by the fact that insulin is already present and active in serum. In contrast figure 3.4.2 indicates that (long-term) insulin treatment does inhibit myofibroblast differentiation in DTTM which again is consistent with similar experiments performed by Linge et al. (2004) and Mackie (2004). More importantly however, these results demonstrate that exogenous TGF- β 1 induces myofibroblast differentiation despite short- (fig. 3.4.1) or long-term (fig. 3.4.2) insulin treatment. Thus insulin does not appear to exert its myofibroblast inhibiting effect in the presence of exogenous (mimicking paracrine) TGF- β . And yet insulin treatment is significantly associated with reduced myofibroblast numbers in the healed scar as show by the murine wound model (Mackie 2004), when paracrine TGF- β would inevitably been present at the time of insulin treatment (wound closure).

These seemingly contradictory findings can be resolved however. The number of myofibroblasts in the scars were assessed at 14 days after the wound was incised closed and treated with insulin in contrast with the assessment of the proportion of myofibroblasts which occurred 4 days after commencement of TGF- β treatment (mimicking wounding) in the cell cultures in figures 3.4.1 and 2. This suggests that insulin does not have antifibrotic action in the presence of active TGF- β but once the first burst of active paracrine TGF- β in the wound have waned insulin is able to exert

its myofibroblast inhibiting effect. Insulin's mode of action therefore must either be a mechanism independent of TGF- β 1 or it inhibits the delayed effects of paracrine TGF- β 1 (after its levels have dropped) such as the production/action/activation of autocrine TGF- β 1.

3.5 Does insulin inhibit the expression of EDA-fibronectin?

3.5.1 Introduction

EDA-fibronectin, mechanical tension and TGF- β 1 are all involved in the induction of myofibroblast differentiation (Grotendorst et al. 2004). Although both EDA-fibronectin and TGF- β 1 must be present in combination the role of wound tension is less clear cut as myofibroblast differentiation is induced *in vitro* in plated cell cultures as is seen in many of the experiments contained within this thesis.

Fibronectin is a 440kD dimeric glycoprotein found throughout the plasma and extracellular matrix and is found in particularly high levels in healing wounds (Kurkinen et al. 1980). It has a repeating motif structure with multiple binding sites for cell-surface receptors and other extracellular matrix components. Fibronectin polymorphism is due to splicing of the RNA to give three splice variants, EDA, EDB and IIICS although an additional splicing variant lacking the IIICS region has also been characterized (MacLeod et al. 1996). Granulation tissue fibroblasts show a splicing pattern consisting of both EDA and EDB but *in vitro* TGF- β 1 preferentially promotes accumulation of the EDA fibronectin isoform (Balza et al. 1988).

EDA-fibronectin expression precedes and its levels are correlated with α -SMA expression in granulation tissue *in vivo* and if it is blocked with antibodies *in vitro* α -SMA expression in fibroblasts is completely prevented even after TGF- β 1 treatment (Serini et al. 1998). Furthermore, the degree of TGF- β 1 induced myofibroblastic

differentiation in cells from a variety of organs is proportional to the levels of EDA-fibronectin those organs produce (Serini et al. 1998). Importantly however, EDA-fibronectin does not by itself stimulate myofibroblast differentiation.

EDA-fibronectin thus provides a mechanism whereby insulin could reduce myofibroblast differentiation directly by inhibiting the expression of just a single protein, even in the presence of activated TGF- β 1. This section aims to indicate whether there is a detectable change in EDA-fibronectin protein expression in insulin treated fibroblasts.

3.5.2 Results

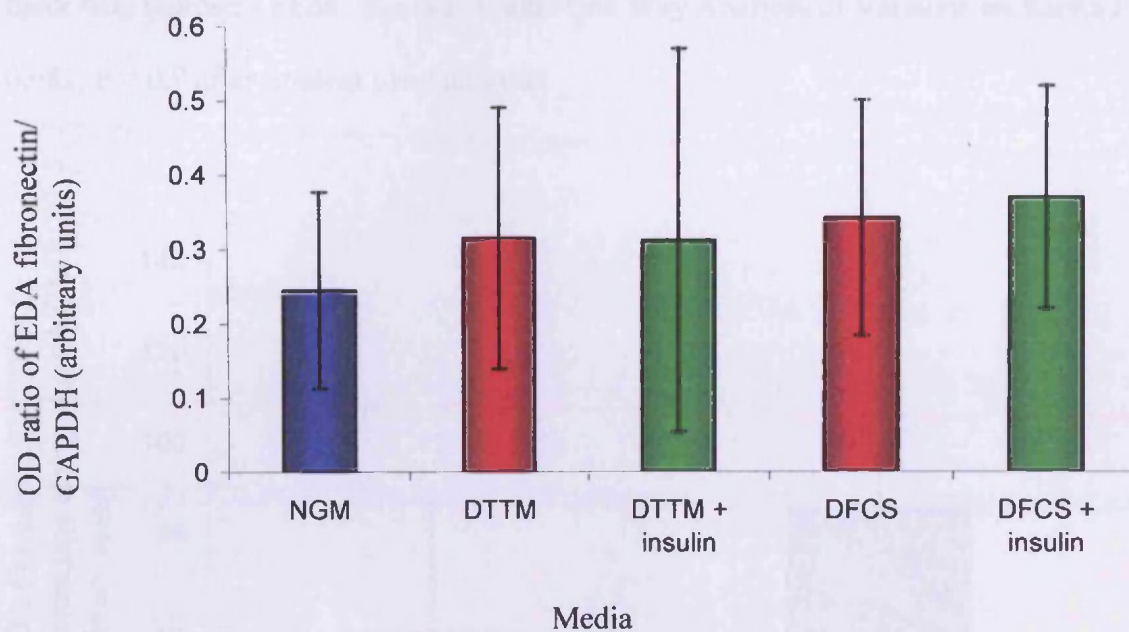
Normal scar fibroblasts were cultured in T25 flasks (7.5×10^5 cells seeded in each flask at day 0) in five different media: DFCS with and without bovine insulin (5 μ g/ml), DTTM with and without bovine insulin (5 μ g/ml) and 10% NGM. After 14 days (the time at which there is maximal myofibroblast induction) cell lysates were prepared from the cultures for SDS PAGE gel electrophoresis and Western blotting (see section 2.5 for technique). Anti-EDA-fibronectin and anti-GAPDH antibodies (see appendix I regarding the use of GAPDH as a housekeeping gene) were used prior to densitometry analysis.

To corroborate the Western blotting findings, immunostaining of fibroblasts seeded on coverslips and treated in the same five media was performed after two weeks culture

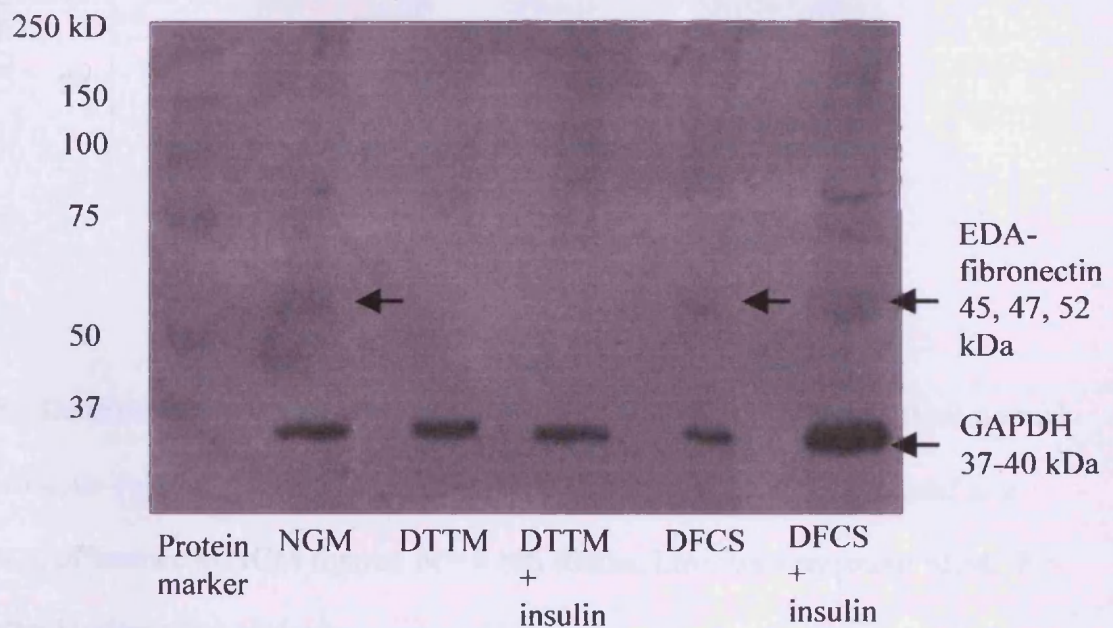
using anti- EDA-fibronectin antibodies and propidium iodide counterstain followed by fluorescence microscopy and photography (see section 2.4).

Insulin treatment does not significantly affect the ratio of protein expression of EDA-fibronectin/GAPDH as measured by Western blot analysis (see figure 3.5.1a). The large standard error bars reveal the considerable variation in EDA-fibronectin expression that was found between cell isolates of different origins, which could obscure any possible trend. However, figure 3.5.1a does show that there is greater EDA-fibronectin expression in those fibroblasts cultured in growth factor depleted/deactivated media in comparison with fibroblasts cultured in NGM, which is in keeping with the higher myofibroblast numbers in these media, although this difference did not quite reach significance. Attempts at normalising the results between cell strains by expressing the results as a percentage increase from the EDA: GAPDH ratios of cells cultured in NGM (see figure 3.5.2) also failed to show any significant difference although did indicate a possible trend towards increased EDA-fibronectin expression in the insulin treated cells (by almost 50%). If this is a real finding it may reflect the overall improved health of the insulin treated cells.

The immunostaining (see figure 3.5.3) reveals uniform EDA-fibronectin expression by every cell in each medium which corroborates the conclusion from the Western blotting experiment that insulin doesn't reduce myofibroblast differentiation by inhibiting EDA-fibronectin expression.



a)



b)

Fig. 3.5.1 a and b. Densitometry analysis of western blots (a) for EDA fibronectin taken from normal scar fibroblasts cultured for 14 days DFCS and DTTM with and without insulin and a representative membrane. b) Representative Western blot showing bands for

EDA-fibronectin at 45, 47 and 52 kDa and the GAPDH at 36 to 40kD. N = 4 cell strains. Error bars represent SEM. Kruskal-Wallis One Way Analysis of Variance on Ranks $P = 0.982$; $P > 0.9$ after Student t-test analysis.

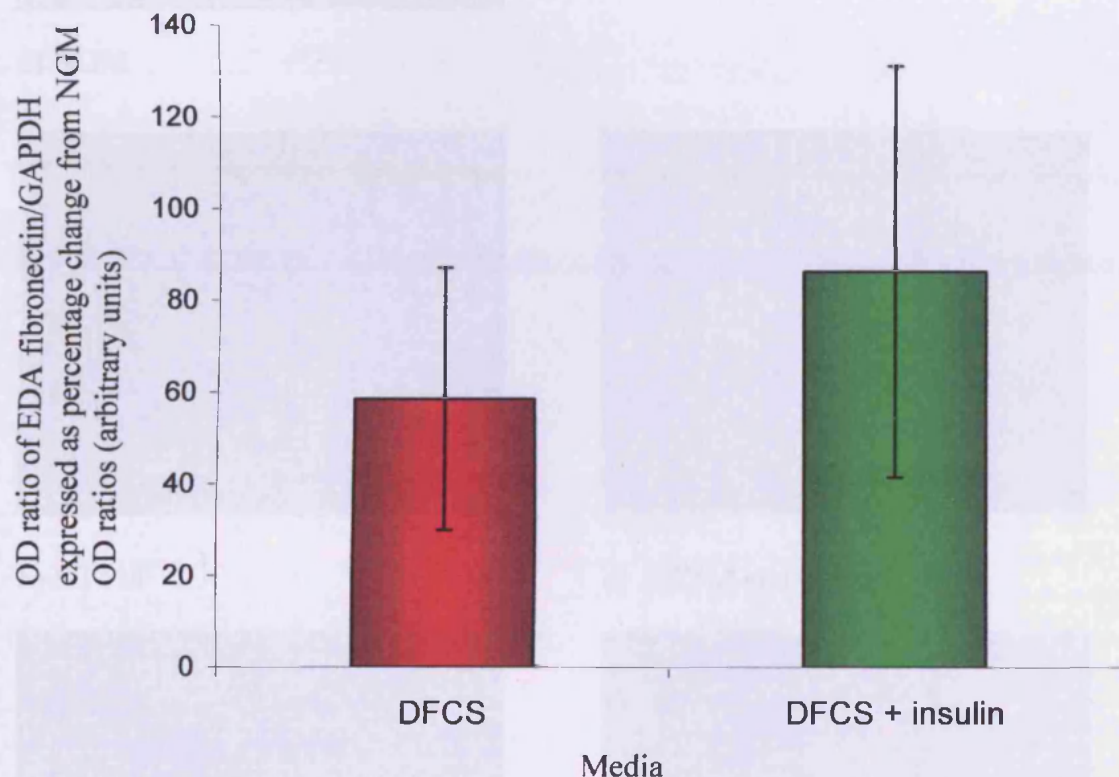
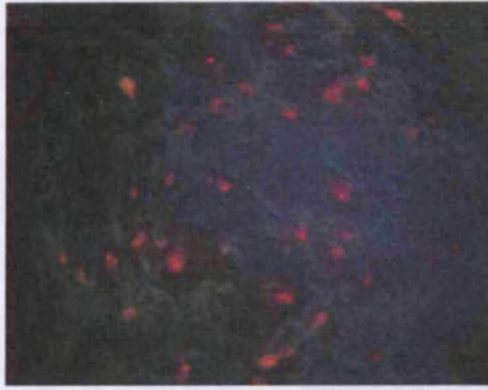
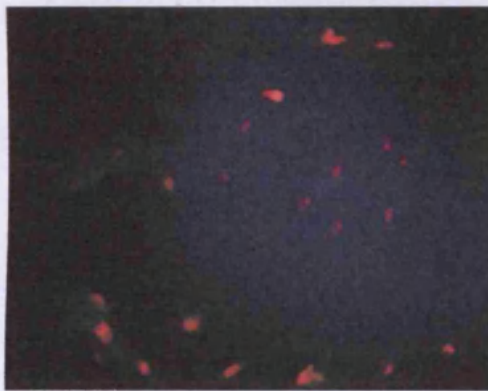


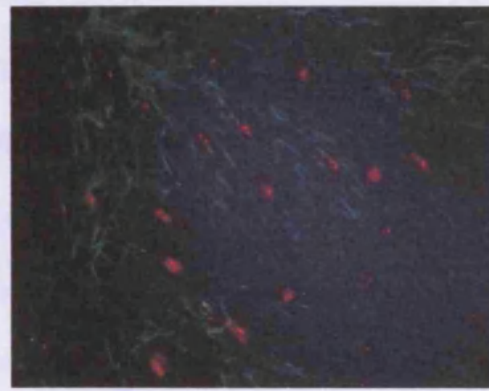
Fig. 3.5.2 Densitometry analysis of western blots for EDA fibronectin taken from normal scar fibroblasts cultured for 14 days in DFCS with and without insulin expressed as a percentage difference to NGM figures. N = 8 cell strains. Error bars represent SEM. $P = 0.604$ after Student t-test analysis.



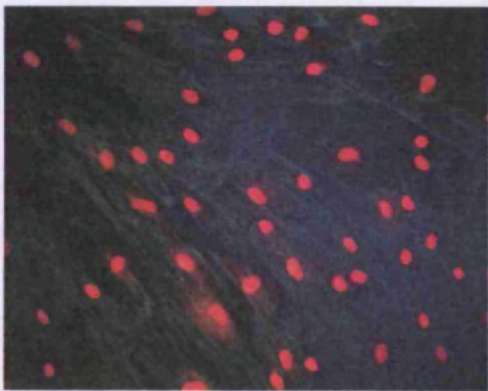
a) NGM



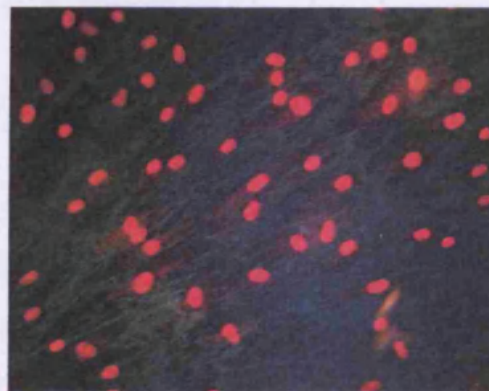
b) DTTM



c) DTTM and insulin



d) DFCS



e) DFCS and insulin

Fig. 3.5.3 Representative micrographs of normal scar fibroblasts which were immunostained with anti-EDA-fibronectin antibodies (FITC conjugated secondary) with a propidium iodide counterstain after 2 weeks culture in five different media: DFCS with and without bovine insulin (5µg/ml), DTTM with and without bovine insulin (5µg/ml) and NGM.

3.5.3 Discussion

The results presented here do not support the hypothesis that insulin's mechanism of action is via the inhibition of expression of EDA-fibronectin. This possibility also seemed unlikely as induction of myofibroblast differentiation by a four day treatment with active TGF- β 1 was not inhibited by long-term pre-treatment with insulin, whereas blockade of EDA-fibronectin is known to inhibit differentiation even in the presence of active TGF- β . However, a blanket inhibition of EDA-fibronectin expression would prevent the appearance of any myofibroblasts or contractile fibroblasts in the wound which could delay wound closure. Furthermore, scarce EDA-fibronectin could also have implications for the strength of the extra cellular matrix because a reduced expression of one splice variant of fibronectin may well affect the balance of expression of other splice variants and impair extracellular matrix assembly. Thus although this would be an effective mechanism of action for insulin, it would perhaps have some undesirable consequences.

3.6 Stress fibres

3.6.1 Introduction

Stress fibres are the fibrillar formation of fibrillar proteins that give myofibroblasts their contractile ability. Stress fibres have important roles in cell adhesion motility and morphogenesis (Thery et al. 2006). They are composed of contractile actin-myosin bundles and there are at least three different types – ventral, transverse and dorsal stress fibres (Hotulainen et al. 2006). There are four main types of myofibroblastic phenotypes that are characterised by the expression of different stress fibre proteins (Skirch et al. 1992) which reputedly represent different stages of progression into the myofibroblast phenotype. These are:

1. vimentin (V type)
2. vimentin and desmin (VD type)
3. vimentin and SM-actin (VA type)
4. vimentin desmin and α -SM-actin (VAD type)

(Desmouliere 1996).

These four types of myofibroblast cannot be distinguished in culture however. Desmin expression is not seen *in vitro*, whereas most dermal fibroblasts express vimentin (C. Linge, personal communication). Nevertheless the presence of stress fibres is crucial to subsequent expression of α -smooth muscle actin, its incorporation into those stress fibres (Ehrlich et al. 2006), and myofibroblast contraction (Hinz et al. 2001).

Examination of myofibroblastic features such as stress fibre formation, EDA-fibronectin α -SMA expression in splinted compared to unsplinted wounds shows that

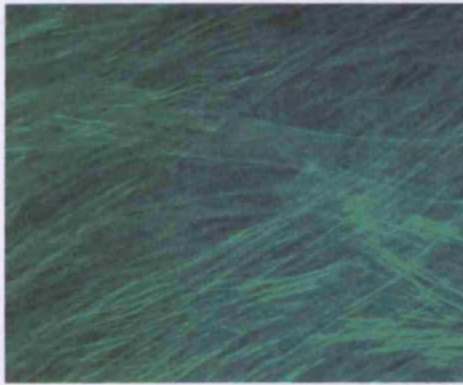
wound tension induces the expression of stress fibres in fibroblasts which allows additional factors, namely TGF- β 1 and EDA-fibronectin, to stimulate the expression of α -SMA (Serini et al. 1998). The expression of this α -SMA subsequently correlates with the degree of focal adhesion maturation and strength of fibroblast adhesion (Hinz et al. 2003).

Thus if insulin were to inhibit the formation of stress fibres it would also inhibit myofibroblast differentiation. This section seeks to test this possibility.

3.6.2 Results

Normal scar fibroblasts were cultured on coverslips in 10% NGM, DFCS with and without insulin (5 μ g/ml), and DTTM with and without insulin (5 μ g/ml). After 14 days the coverslips were fixed in methanol and stained with phalloidin (Sigma-Aldrich[®]). Phalloidin is a toxin from the toadstool "Death Cap" (*Amanita phalloides*) that binds F-actin (L6w & Wieland 1974) and can be used to demonstrate actin structure within cells.

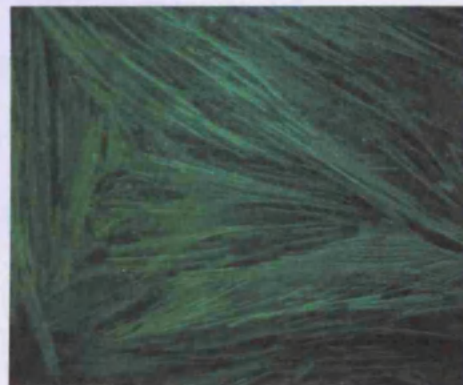
As figures 3.6.1 a to e show, fibroblasts cultured in all of the media with and without insulin all demonstrate stress fibre expression. All cells show well defined stress fibres and there is no appreciable difference in the intensity or pattern of staining of the cells in the different media. Therefore, insulin does not appear to inhibit the formation of stress fibres.



a) NGM



b) DFCS



c) DFCS plus insulin



d) DTTM



e) DTTM plus insulin

Fig. 3.6.1 Representative phalloidin staining of normal scar fibroblasts cultured for 14 days in a) NGM, b) DFCS, c) DFCS plus insulin (5µg/ml), d) DTTM and e) DTTM plus insulin (5µg/ml). (40 x).

3.6.3 Discussion

This experiment indicates that insulin does not inhibit the formation of stress fibres and therefore it is unlikely that insulin inhibits the early stages of myofibroblast differentiation. Phalloidin stains all stress fibres therefore it is not possible to ascertain from this experiment whether the individual types of stress fibres are being formed normally or whether the formation of some is in fact depressed. Nevertheless, the micrographs do demonstrate the typical appearance of stress fibres. It is perhaps fortunate that insulin does not inhibit stress fibre formation, as contraction by stress fibres helps to close wounds. However, although the stress fibres seem unaffected by insulin treatment, their mechanical function is dependent on other factors such as FAKinase (see section 3.7).

3.7 FAK Kinase and phosphorylated FAK Kinase

3.7.1 Introduction

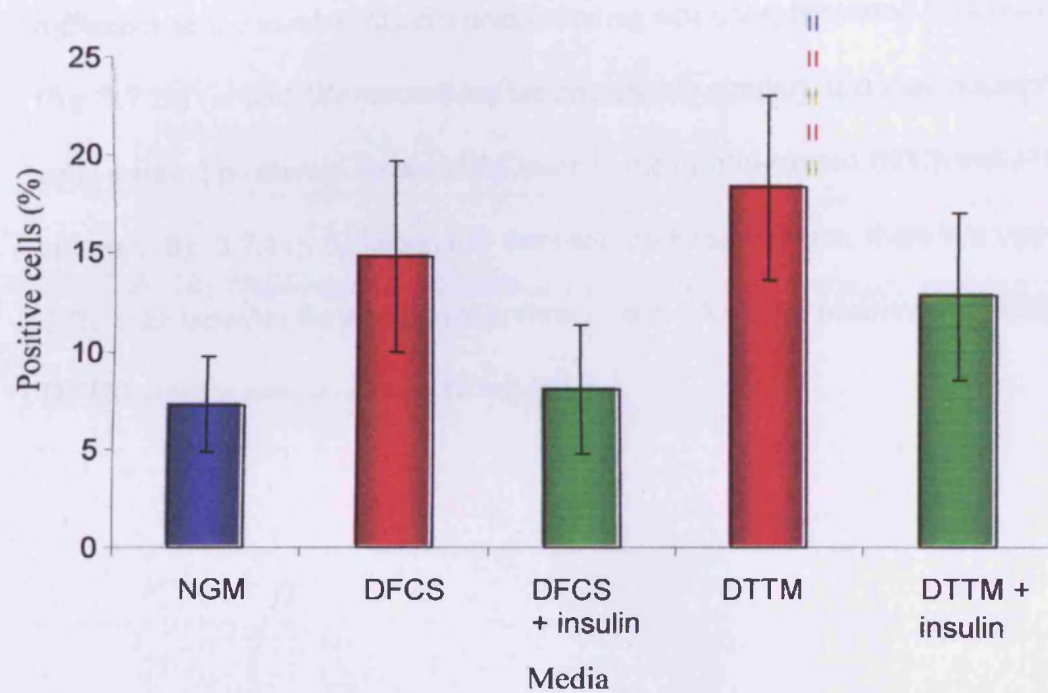
Focal adhesions are molecular complexes that mediate the regulatory effects of the extracellular matrix adhesion on cell behaviour (Chen 2003). They have a mechanical function in that they serve as links between the cell and extracellular matrix and are the sites of stress fibre assembly (Endlich et al. 2007). They also have roles as biochemical signalling centres at sites of integrin binding (Alam et al. 2007). Focal adhesions convey messages about the condition of the ECM to the cell thus affecting its behaviour. Focal adhesions contain over 50 proteins. Central to the mechanosensory role of focal adhesions is focal adhesion kinase (FAK) which is an enzyme recruited by adhesion-dependent activation of integrins within the focal adhesion complexes (Schaller et al. 1992; Giancotti et al. 1999). Activation of FAK is via SRC kinase, which induces the phosphorylation of tyrosine 861 within the FAK COOH terminus, which facilitates the association of FAK with integrin $\alpha v \beta 5$ (Eliceiri 2006). FAK activation is in the form of auto phosphorylation at tyrosine 397 and this is induced after a delay of hours (suggesting a requirement for new protein synthesis) by TGF- $\beta 1$ (Hinz et al. 2003). Once activated, FAK can recruit SRC to focal adhesions, leading to ERK activation (Courtneidge et al. 1993; Aplin et al. 1998) and also promotes downstream integrin mediated signals (Parsons et al. 1997). In addition to SRC, several other related molecules associate with FAK, including paxillin (Turner et al. 1994), PAI3 kinase (Chen et al. 1994) and Grb2 (Schlaepfer et al. 1994).

Importantly, pharmacologic inhibition of FAK kinase by PP2 inhibits the TGF- β 1-induced myofibroblast differentiation in a dose-dependent manner (Thannickal et al. 2003) and fibroblasts expressing kinase deficient FAK are also unable to differentiate into myofibroblasts. Therefore, if insulin were to effect the expression or phosphorylation of FAK kinase, it could block TGF- β 1 induction of myofibroblast differentiation. This section aims to see whether this could possibly be insulin's mechanism of action.

3.7.2 Results

Normal scar fibroblasts were cultured on cover-slips in six-well plates for 2 weeks and then fixed in 10% paraformaldehyde. Selections of culture media were used: DFCS with and without bovine insulin (5 μ g/ml), DTTM with and without bovine insulin (5 μ g/ml) and NGM. The coverslips were then immunostained with anti-FAK and anti-phosphorylated FAK antibodies (R & D Systems). Propidium iodide was used as a nuclear counter-stain.

Immunostaining with the anti-FAK and anti-phosphorylated FAK antibodies yielded faint staining of cells in all media tested. Those fibroblasts that did stain positively revealed a faint homogenous cytoplasmic staining. The positive cells were counted and expressed as a percentage of the total. Representative images of insulin-treated and untreated fibroblasts immunostained with anti-FAK and anti-phosphorylated FAK are shown in figures 3.7.1a and 3.7.2a.



c)

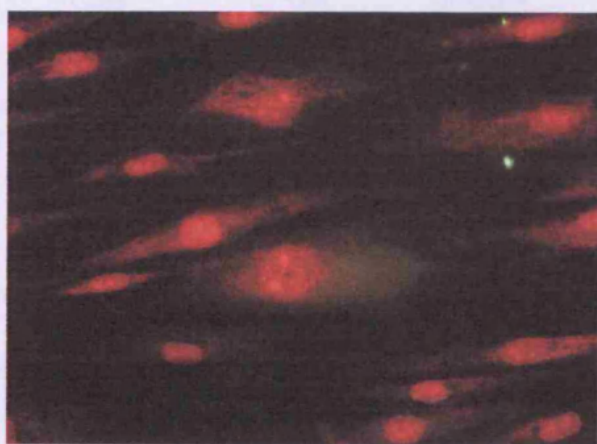
Fig. 3.7.1 a - c. Representative images of normal scar fibroblasts cultured for 14 days in DTTM a) with and b) without insulin (5 μ g/ml) immunostained with anti-FAK kinase antibody with propidium iodide counterstain. c) The percentage of normal scar fibroblasts staining positively with anti- FAK antibodies. N = 3 cell strains. Error bars represent SEM. Kruskal-Wallis one way analysis of variance on ranks $P = 0.042$. Student t-test – DTTM compared with NGM $P < 0.05$, t-tests for remaining comparisons $P > 0.05$.

The results indicate greater numbers of fibroblasts expressing both FAK and phosphorylated kinase in the growth factor deactivated media (DTTM) which is in keeping with the finding that this media generally leads to the highest percentages of myofibroblasts in fibroblast cultures. Whereas insulin treatment is not associated with a difference in the number of cells demonstrating anti-phosphorylated FAKinase staining (fig. 3.7.2a) (in fact, the proportions are remarkably similar), there are noticeably fewer cells staining positively for anti-FAKinase in the insulin-treated DFCS and DTTM cultures (fig. 3.7.1a). Although this does not reach significance, there is a significant difference between the highest proportion of anti-FAKinase positively staining cells in DTTM and the lowest – NGM ($P < 0.05$).

a)



b)



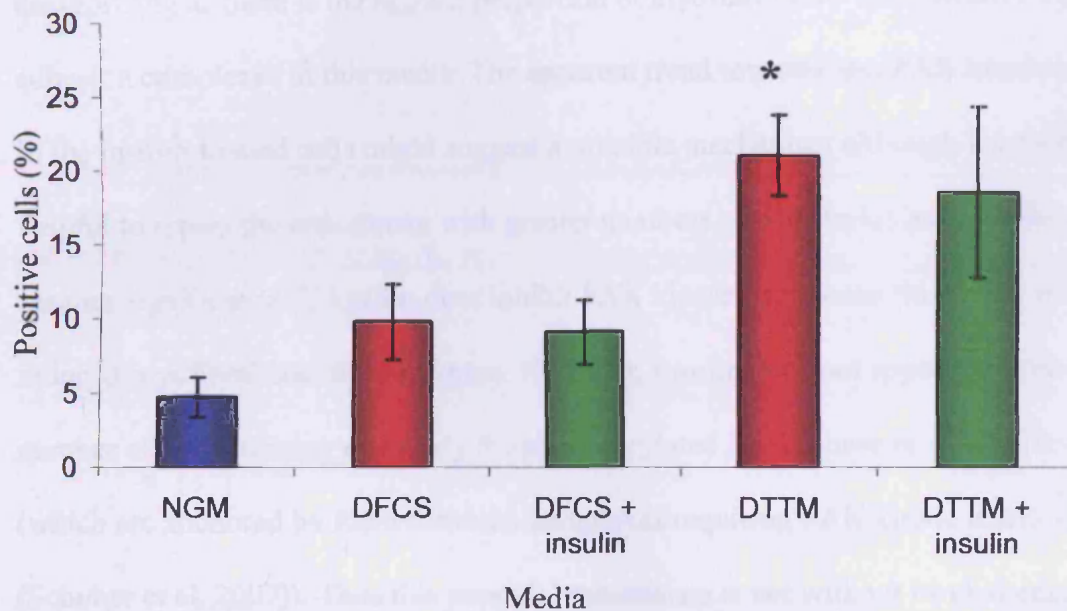


Fig. 3.7.2 a- c. Representative images of normal scar fibroblasts cultured for 14 days in DTTM a) with and b) without insulin (5 μ g/ml) immunostained with anti-phosphorylated FAK kinase antibody with propidium iodide counterstain. c) The percentage of normal scar fibroblasts staining positively with anti-phosphorylated FAK antibodies. N = 3 cell strains. Error bars represent SEM. Kruskal-Wallis one way analysis of variance on ranks $P = 0.019$. Student t-test – DTT (*) compared with NGM $P < 0.05$, t-tests for remaining comparisons $P > 0.05$.

3.7.3 Discussion

The finding that higher numbers of cells stain positively for FAK kinase and phosphorylated FAK kinase in the cells cultured in growth factor deactivated media is unsurprising as there is the highest proportion of myofibroblasts and therefore focal adhesion complexes in this media. The apparent trend towards less FAK kinase staining in the insulin-treated cells might suggest a possible mechanism although it would be helpful to repeat the experiment with greater numbers of cell strains to see if this trend reaches significance. If insulin does inhibit FAK kinase expression this could lead to reduced myofibroblast differentiation. However, insulin does not appear to affect the number of cells staining positively for phosphorylated FAK kinase or stress fibres (which are anchored by focal adhesion complexes requiring FAK kinase activity (Schober et al. 2007)). Thus this potential mechanism is not without its challenges. Attempts at Western blotting for FAK kinase phosphorylated FAK kinase were unsuccessful and abandoned due to time constraints.

3.8 *Thy-1*

3.8.1 Introduction

Thy-1 is a cell surface receptor expressed by proportion of fibroblasts in the human myometrium and orbit and appears to be essential for subsequent myofibroblast differentiation in certain tissues (Koumas et al. 2003). Thy-1 negative fibroblasts are unable to differentiate into myofibroblasts and preferentially differentiate into lipofibroblasts (Koumas et al. 2003). This divides the fibroblast population into two distinct subsets with different differentiation potential. Such fibroblast heterogeneity has been previously shown with regard to proliferation rates, responses to prostaglandin E2, collagen synthesis, cytokine production, cytokine receptors and C1q receptors (Schneider et al. 1977; Botstein et al. 1982; Korn et al. 1984; Jordana et al. 1988; Derdak et al. 1992; Borrello et al. 1996). In addition to the differential functional attributes there is also a diversity of phenotypes within fibroblast populations in terms of morphology and cell size (Kunz-Schughart et al. 2003). Although Thy-1 dependent myofibroblast differentiation has not been examined in dermal fibroblasts, the homology between fibroblasts in different tissues suggests that it is reasonable to examine for this antigen in this research. If insulin can change the Thy-1 status of fibroblasts (from Thy-1 positive to negative), this might explain its inhibition of the differentiation of myofibroblasts. The purpose of this section is to see whether insulin does have this property.

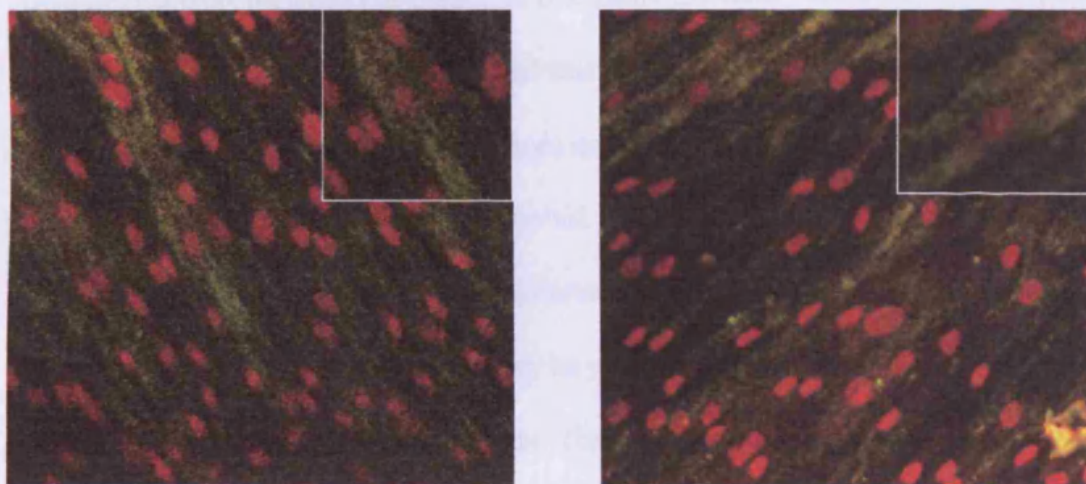
3.8.2 Results

Normal scar fibroblasts were cultured on coverslips in 10% NGM, DFCS with and without insulin (5 μ g/ml), and 4% DTTM with and without insulin (5 μ g/ml). After 14 days the coverslips were fixed in methanol and stained with anti-Thy-1 antibodies (Sigma[®]) and a propidium iodide counterstain and then examined under a confocal microscope (Leica[®]) because of the relatively faint staining.

Fibroblasts stained positively for Thy-1 in all of the media with virtually all cells showing homogenous staining throughout the cytoplasm (see fig. 3.8.1). Insulin does not appear to affect the expression of Thy-1 by fibroblasts.

3.8.3 Characterization

This section characterizes the cells that are present in the scar tissue. The cells are characterized by their morphology, their ability to form colonies, and their ability to produce extracellular matrix. The cells are characterized by their morphology, their ability to form colonies, and their ability to produce extracellular matrix. The cells are characterized by their morphology, their ability to form colonies, and their ability to produce extracellular matrix.



a) Untreated

b) Insulin treated

Figure 3.8.1 a and b. Representative images from confocal microscope of normal scar fibroblasts cultured for 14 days in DFCS and then immunostained with anti-Thy-1 antibodies and a propidium iodide counterstain. Higher magnification images inset. N = 4.

3.8.3 Discussion

These results show that insulin does not have an obvious effect on Thy-1 expression and therefore does not exert its myofibroblast inhibitory affect by down-regulating Thy-1 expression. However, this hypothesised mechanism is based on the assumption that Thy-1 status influences myofibroblast differentiation in the dermis when in fact it currently remains uncertain whether this is actually the case. Nevertheless it is clear that almost all cells of each of the normal scar strains expressed Thy-1. Attempts at corroborating the staining results in a more quantitative manner failed and Western blotting of this protein could not be achieved.

Although the results indicate the straightforward conclusion given above, it may be misleading, since Thy-1 distribution may be just as important and lastly insulin could affect Thy-1 in terms of its conformation. Thy-1 resides on the cell surface and apparently does not associate with itself or other peptides, perhaps in a immunological role (Abeysinghe et al., 2003), but if in future research an interaction is found then insulin's possible influence here would need further exploration.

3.9 Does insulin stimulate fibroblast differentiation into a lipid-expressing phenotype?

3.9.1 Introduction

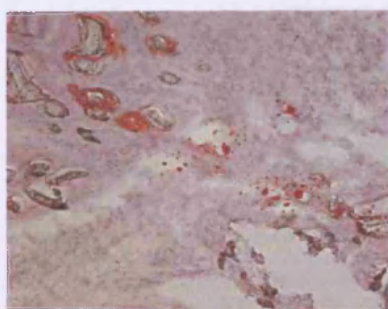
Insulin is an anabolic hormone and stimulates adipogenesis in many tissues. This can even go so far as to cause differentiation into adipocytes. To the author's knowledge human fibroblasts have never been demonstrated to differentiate into adipocytes; however, the true nature of fibroblastic cells which migrate into the wound area, take part in the healing process and form scar tissue is uncertain, with different groups claiming derivation from pericytes, adipocytes and blood borne pluripotential cells (see section 1.4.3). Fibroblastic pre-adipocytes in rats are known to differentiate into adipocytes (Mitchell et al., 1997) while mouse fibroblasts have been shown to differentiate into adipocytes in response to troglitazone, an agent that decreases insulin resistance in diabetics (Liu et al. 2004). In addition, human fibroblasts express both insulin and IGF receptors and respond with the same spectrum of metabolic and mitogenic actions as myoblasts and adipocytes (Niesler et al. 2001; Siddle et al. 2001; Urso et al. 2001)). The combination of these facts suggests a possible mechanism for reduced myofibroblast numbers in insulin-treated fibroblast cultures – that is, insulin, rather than inhibiting myofibroblast differentiation, instead promotes fibroblasts differentiating into adipocytes or simply accumulating lipids rather than α -SMA. This would have the effect of reducing the proportion of myofibroblasts in a wound and subsequent scarring but with a possible disadvantage of a probably weaker wound (more likely to lead to a stretched scar). The purpose of this section is to see whether

insulin treatment is associated with an accumulation of adipocytes in fibroblast cultures.

3.9.2 Results

Normal scar fibroblasts were cultured for 14 days on coverslips in NGM, dialysed FCS and DTTM with and without insulin (5µg/ml). After paraformaldehyde fixation the coverslips were stained with oil red O (which stains lipids red) and a haematoxylin counterstain.

The positive controls (skin) shows several adipocytes which are large, globular and stained homogenously and intensely red throughout the cytoplasm (figures 3.9.1 a), whereas the pre-adipocytes (derived from human adipose tissue – a kind gift from C. Linge) after insulin treatment showed numerous smaller lipid droplets (fig. 3.9.1f). In contrast, despite a scattering of oil red O staining in the fibroblast cultures (particularly in DTTM), there are no cells that resemble adipogenic phenotypes (figs. 3.9.1b-c). Insulin treatment does not appear to be associated with increased oil red O staining in normal scar fibroblast cultures.



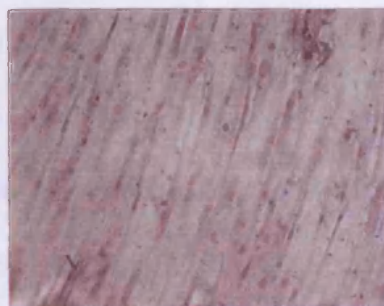
Positive control: skin



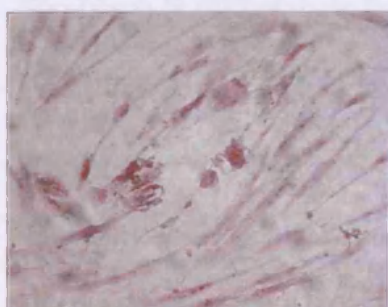
a) NGM



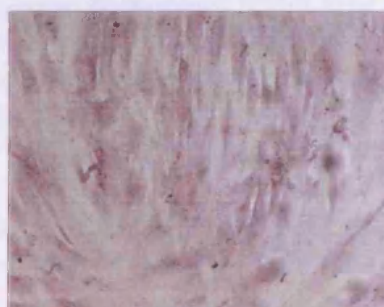
b) DFCS



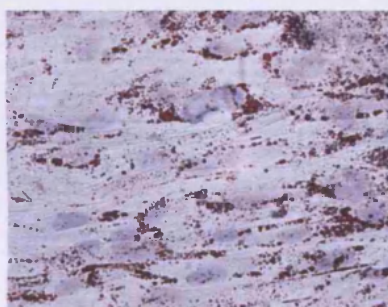
c) DFCS plus insulin



d) DTTM



e) DTTM plus insulin



f) Preadipocytes plus insulin.

Fig. 3.9.1 a-f. Typical appearances of normal scar fibroblasts stained with oil red O and a haematoxylin counterstain after 14 days culture in a) 10% NGM, b) DFCS with and c) without insulin, d) DTTM with and e) without insulin (5 μ g/ml). F) shows preadipocytes for comparison.

3.9.3 Discussion

Clinical experience is in agreement with the findings of this experiment. Insulin is known to speed the healing of diabetic ulcers and repeated subcutaneous injections to the same site are known to cause both fat hypertrophy and atrophy but is not known to cause true fibroblasts to differentiate into adipogenic phenotypes. Nevertheless, lipogenesis of the fibroblastic pre-adipocytes in cultures is induced by insulin (see fig. 3.9.1 f). However, with evidence that insulin may induce such changes in other species (Liu et al. 2004) it is important to rule out such an affect in humans too.

The results from this experiment indicate that insulin does not cause reduced myofibroblast numbers in growth factor deactivated/depleted media by stimulating fibroblasts to differentiate into adipogenic phenotypes rather than myofibroblasts. This is an important finding as although such a mechanism is potentially viable an increased number of adipocytes in a wound in relation to myofibroblasts could possibly result in a weaker wound. This would clearly be an undesirable mechanism of action for insulin as an anti-scarring agent.

3.10 Discussion and Conclusion

The chapter began with the reproduction of the original experiments that showed increased myofibroblast differentiation in growth factor deactivated media compared to NGM (Linge 2004) albeit with fibroblasts derived from normal scar rather than dermis. The reproducibility of these findings is important for giving credence to subsequent experiments and for honing the techniques required for the rest of the thesis. Interestingly however, there is a clear and consistent difference in the maximal proportion of myofibroblasts reached with Mackie (2004) reporting that myofibroblasts comprised 10% of dermal fibroblast cultures at 14 days whereas the results presented in this thesis (see fig. 3.2.2 and other figures) yielding approximately 25% myofibroblasts in normal scar cultures. This marked difference could be due either to possible differential sensitivities to the inhibitory effects of the dithiothreitol-treated serum which is toxic at higher levels or due to intrinsic differences in capability for myofibroblast differentiation between normal scar and dermal fibroblast derived lineages. Perhaps normal scar fibroblasts, due to their derivation from a scar-inducing cytokine environment, are more 'primed' to differentiate into myofibroblasts given the right conditions. In contrast, this difference is not seen in 10% NGM, both dermal and normal scar fibroblast cultures yielding roughly 5% myofibroblasts at day 14 (see fig. 3.2.2).

The findings of this first experiment (see fig. 3.2.2) were not limited to the differences in myofibroblast numbers. In addition it was noted that the intensity of α -SMA

immunostaining observed under the microscope showed considerable variation. In a single culture, even within a single field of view, there was a spectrum of intensities; however the faint staining fibroblasts were mostly confined to the fibroblasts that had been cultured in dialyzed FCS, whether DTT-treated or not rather than in NGM whereas the more intense staining cells (although fewer in number) were common to all cultures irrespective of medium used. Perhaps these intense staining myofibroblasts are a sub-population that are refractive to insulin treatment and their high levels of alpha-SMA expression may go part way to explaining finding by Mackie (2004) of an apparently greater inhibition of alpha-SMA expression by insulin detected by immunohistochemistry as opposed to western blotting.

It has been assumed that the reduction in the proportion of myofibroblast in insulin-treated cultures is due to inhibition of myofibroblast differentiation but there is a possible alternative mechanism that is worth noting. It has been recorded in animal studies that myofibroblasts disappear from scars when they are beginning to mature (Vande Berg JS et al, 1985) and myofibroblasts have also been seen to apoptose towards the end of wound healing (Desmouliere et al. 2005). Thus rather than preventing differentiation of myofibroblasts, insulin may cause apoptosis of myofibroblasts or even de-differentiation. However, the former scenario seems unlikely as no dead or dying cells nor obvious apoptotic bodies were evident throughout the time course of the experiments.

The proliferation assay (fig. 3.3.2) showed a trend towards increased fibroblast proliferation with insulin treatment but this did not reach significance. Insulin is both a

survival factor, or apoptosis inhibitor (Prisco et al. 1999) and a mitogenic factor (Krupsky et al. 1996) with efficacy demonstrated in a number of fibroblast cell lines (Khil et al. 1997), (Gonzalez-Hernandez et al. 1993), (Selgas et al. 1989). This effect is seen to a degree in the assay (see fig. 3.3.2) but as this doesn't reach significance it is difficult to pose selective proliferation of fibroblasts as the reason for reduced myofibroblast numbers following insulin treatment. This makes it more likely that insulin exerts its effect on the differentiation of the myofibroblast phenotype or, less likely, the de-differentiation from myofibroblast to fibroblast. Nevertheless, it is important to be aware of the increased proliferation associated with insulin treatment as it could influence results. For example, in subsequent Western blot experiments an increased number of cells in the insulin treated samples would mean generally higher protein levels. To control for this discrepancy a constitutively expressed protein (GAPDH) must also be blotted and used for comparison.

The proliferation assay (fig. 3.3.2) also showed a greater proliferative effect of insulin in NGM than in DFCS or DTTM. This is in spite of the fact that NGM already contains insulin. This could be because the paucity of growth factors in the dialysed FCS impairs the ability of the fibroblasts to respond to the mitogenic effects of insulin. The increased proliferation with the addition of insulin indicates that the levels of insulin in FCS below those required for maximal response. It is also a reassuring sign that the bovine insulin being used in this study and at this particular concentration is active and effecting the cells.

The proliferation assay also suggests that dialysis of FCS (DFCS) and even DTT-treatment (DTTM) still allows fibroblasts to proliferate well beyond 14 days. This is an important finding for those experiments that involve time courses beyond 14 days but also suggests that even DTT is not as free of growth factors as supposed. However, with the enormous number of growth factors known and unknown in FCS, performing specific growth factor assays would be an impractical undertaking.

Section 3.4 began the examination of one the main potential mechanisms of insulin's action, namely one involving TGF- β 1. But the experiment showed (see fig. 3.4.1) that after prolonged (14 days) insulin treatment there was no significant inhibition of the increase in the myofibroblast numbers induced by exposure to exogenous active TGF- β 1. This indicates that insulin does not prevent the action of active TGF- β 1, hence the investigation into other possible mechanisms. However, this does not mean that TGF- β 1 is not involved – insulin may still affect the autocrine TGF- β 1 that is secreted by fibroblasts in an inactive form. This will be discussed in greater detail in chapters 4 and 5.

The remaining sections of this chapter examined those potential mechanisms of action of insulin that do not involve TGF- β . Taken together these sections suggest that insulin does not inhibit the differentiation process grossly via alteration of a range of factors required for the successful differentiation of fibroblasts into myofibroblasts nor does it direct the differentiation process down the alternate route of adipogenesis. Neither EDA-fibronectin nor stress fibres, both prerequisites to the induction of myofibroblast

differentiation, demonstrate altered expression/formation in response to insulin treatment. The expression of other proteins such as FAKinase, phosphorylated FAKinase (section 3.7) and Thy-1 (3.8), involved in the differentiation process are also unaffected by insulin. Furthermore, insulin does not induce adipocyte differentiation in fibroblasts.

The expression of the majority of these proteins has only been examined in a rather crude manner (immunohistochemical staining) due to technical difficulties with Western blotting for some of these antigens combined with time constraints. However, the consistent absence of any clear change of multiple inter-related factors (in response to insulin) consolidates each of the individual findings suggesting that they do not play a significant role in the highly significant reduction of α -SMA positive myofibroblast phenotype caused by insulin. Therefore, if significant inhibition of any one of these proteins were found in response to insulin we might expect to see some sort of effect on the other proteins, for example if EDA-fibronectin expression was reduced by insulin then it would be logical for subsequent stress fibre production and FAKinase phosphorylation to be reduced too. Furthermore, if insulin were to inhibit the expression of these essential factors, a deleterious effect on wound healing might be expected, but as has been previously demonstrated in the murine wound model (Mackie, 2004), this is not the case. This effect of insulin cannot simply be on the expression of the α -SMA gene itself nor can it be directly on a late stage of the differentiation process because TGF- β can still induce α -SMA positive myofibroblasts in cultures that have received prolonged treatment with insulin.

The finding that insulin did not promote differentiation of fibroblasts into an adipogenic phenotype (fig. 3.9.1) rules out this mechanism thereby answering a potential criticism of insulin as an anti-scarring agent. Interestingly, it might also indicate that “scar fibroblasts” are not simply de-differentiated adipocytes, although it is possible that the de-differentiation process is irreversible. In addition, insulin does not seem to affect Thy-1 which may determine a fibroblast’s potential to differentiate into a myofibroblast.

This chapter has necessarily relied heavily on immunostaining which as a technique has some flaws. The difficulty with assessing protein expression in this way is that it is dependent on the protein being present in the cells in sufficiently high levels for the subsequent fluorescent staining to be detectable. If the proteins are expressed at relatively low levels then the incidence of false negatives will be higher. The judgement of ‘positivity’ is necessarily subjective and in those experiments where the proportion of positively staining cells was very low it is difficult to draw valid conclusions. Examining the proteins with SDS PAGE and immunoblotting and densitometric quantification is clearly the best way to clarify this and this has been attempted for EDA-fibronectin, FAK kinase and phosphorylated FAK kinase. However, the work contained in this chapter was performed in parallel with the investigations into the role of TGF- β in relation to insulin’s myofibroblast inhibitory effects (see chapters 4 and 5). As these experiments demonstrated greater potential, later efforts were directed towards them rather than the continuation of the investigations in this chapter.

3.11 Conclusion

1. Myofibroblast differentiation of normal scar-derived fibroblasts is increased in growth factor deactivated media (DTTM) compared to NGM and this is demonstrable in the author's hands (fig. 3.2.2).
2. There is no significant difference in the proliferation of fibroblasts in NGM or DTTM and DFCS with or without insulin (fig. 3.3.2). Therefore differences in proliferation between the different media are unlikely to be a source of confounding in the experiments contained in this and the following chapters.
3. Neither short- (4 days) (fig. 3.4.1) nor long-term (14 days) (fig. 3.4.2) insulin treatment prevents the increase in myofibroblast numbers that is induced by exogenous TGF- β 1 treatment indicating that insulin does not prevent the action of active TGF- β 1.
4. Insulin does not inhibit the expression by fibroblasts of EDA fibronectin (figs. 3.5.1-3), stress fibres (fig. 3.6.1), FAKinase nor does it affect the phosphorylation of FAK (figs. 3.7.1 and 2). This suggests that insulin does not inhibit the myofibroblast differentiation process via several of the factors reported to be required for successful differentiation.
5. Insulin does not inhibit affect the expression of Thy-1 which may determine a fibroblast's potential to be able to differentiate into a myofibroblast nor does it stimulate fibroblasts to differentiate into adipocytes (fig. 3.9.1).

Chapter 4

**Is insulin's effect on
myofibroblast differentiation
via transforming growth factor
 β ?**

4.1 Introduction

The results of the previous chapter show that neither brief nor prolonged treatment with insulin renders fibroblasts incapable of differentiating into myofibroblasts in response to exogenous active TGF- β 1 (see figs. 3.4.1 & 2). Therefore if active TGF- β is present, myofibroblast differentiation occurs despite any form of treatment with insulin. Thus an alternative theory for the mechanism of insulin's action is that it acts through effects on autocrine TGF- β in some way, either through absolute or relative levels of each TGF- β isoform. In the context of this theory therefore it is of interest to examine the effects of insulin on fibroblasts derived from early gestational age (< 14 weeks – non-scarring phenotype) fetal fibroblasts since they do not appear to have the autocrine loop of TGF- β gene expression exhibited by the more developmentally mature cells.

Transforming growth factor beta is the major cytokine central to scarring. It is released by platelets, macrophages and a host of other cells at the time of wounding and acts on fibroblasts to stimulate differentiation into myofibroblasts as well as production of autocrine TGF- β 1 by the same fibroblasts (Liu et al. 2004). As detailed in section 1.5.2, TGF- β has three isoforms that are important in scarring. TGF- β 1 and 2 are pro-fibrotic and TGF- β 3 is antifibrotic (Shah et al. 1992). Of the three, TGF- β 1 is the only isoform expressed in platelets and takes the dominant role, the other two isoforms being far less abundant (Liu et al., 2004).

Transforming growth factor was first described in 1981 as a growth factor that acted in an autocrine fashion and was also capable of inducing a transformed morphology (Moses et al. 1981). Evidence of the importance of TGF- β 1 expression in wound healing came in 1987 when Cormack demonstrated time-dependent TGF- β expression in wound fibroblasts in subcutaneous wound chambers in rat skin (Cormack 1987). TGF- β 's potential became clear when it was found to increase the tensile strength of rat incisional wounds and accelerate wound healing (Mustoe et al. 1987).

It wasn't until the early nineties that the implications of the relative expression of TGF- β 1, 2 and 3 were uncovered with the experiments of Shah et al (Shah et al. 1992; Shah et al. 1994; Shah et al. 1995). TGF- β 1 and 2 neutralising antibodies applied to full thickness incisional rat wounds led to reductions in inflammatory cell infiltration, neovascularisation and deposition of collagen types 1 and 3, when compared to control wounds (Shah, Foreman et al. 1992 & Shah, Foreman et al. 1994). Furthermore, the resulting dermal architecture of the treated wounds resembled unwounded skin rather than scar tissue and yet, interestingly, antibodies to solely either TGF- β 1 or 2 did not have the same anti-scarring effect as the addition of both antibodies together (Shah, Foreman et al. 1994). In addition, exogenous TGF- β 3 administered to wounds produced the same effects as the TGF- β 1 and 2 blocking antibodies (Shah et al. 1995). More recently, raised TGF- β 1 expression has been implicated in hypertrophic (Wang et al. 2000) and keloid scarring (Lee et al. 1999). Conversely, various mechanisms of TGF- β 1 under-expression may underlie the scarless healing of fetuses (Soo et al. 2000; Hsu et al. 2001; Rolfe et al. 2007b).

The importance of TGF- β 1 protein expression in scarring is suggested by the finding that raised TGF- β 1 (and 2) levels are found in keloids compared to normal fibroblasts (Lee et al. 1999) and that TGF- β 1 protein levels are approximately 30% higher in scarring postnatal skin compared to fetal scarless healing skin (Chen et al. 2005). Neutralising TGF- β 1 in wounds with antibodies improves scarring (Shah et al., 1992; Shah et al., 1994) and TGF- β 1 antagonists such as decorin can inhibit its fibrotic effects (in glomerulonephritis) (Border et al. 1992). These findings of Shah et al have led to proposals from the company Renovo Ltd of using neutralising antibodies as anti-scarring therapies (Ferguson et al., 2004). However, there are problems with antibody therapy such as their prodigious expense and short half-lives in wounds, in addition to the problem of immune responses being generated at the wound sites. A possible alternative solution comes from gene therapy. A self-protein gene can be introduced into the wound, which can sustain physiological doses of the protein in question for a relatively long period; furthermore, such a self-protein would be non-immunogenic and could be used repeatedly. For example, Isaka et al introduced the decorin gene to animal models and demonstrated that the resulting expression of decorin could prevent renal fibrosis due to its neutralising effect on TGF- β activity (Isaka et al. 1996). Choi et al decreased TGF- β 1 production in wounds with an antisense oligonucleotide approach and markedly reduced scarring in a healed wound (Choi et al. 1996). These gene therapies have focused on TGF- β 1 but gene therapy could equally apply to TGF- β 2 and 3, that is, decreasing the expression of the former and increasing the expression of the latter.

In the discussion of the TGF- β isoform profile during wound healing it must be remembered that TGF- β 1 comes from two sources: a paracrine source from platelets and inflammatory cells and an autocrine source from the wound fibroblasts themselves. Insulin could potentially only affect one of these sources. In the murine wound model used by Ian Mackie (2004) insulin was administered once at the time of wound closure and found to significantly affect myofibroblast numbers 14 days later, after much of the paracrine TGF- β 1 had dissipated thus suggesting that insulin does not exert its effect by inhibiting the production of paracrine TGF- β 1 (Mackie, 2004). In addition, it has already been demonstrated that prolonged insulin treatment does not prevent fibroblasts differentiating into myofibroblasts in response to exogenous TGF- β 1 treatment (mimicking wounding and the release of paracrine TGF- β 1, see section 3.4) Conversely, the basal proportion of myofibroblasts in fibroblast cultures from normal skin, normal scar, hypertrophic scar and keloids appears to correlate with the levels of autocrine TGF- β 1 secreted by those cells (Linge et al. 2004). Therefore, could insulin instead affect the expression or action of autocrine TGF- β 1?

The manipulation of the TGF- β isoform profile in healing wounds, particularly in relation to TGF- β 1, is also a credible means of inhibiting scar formation. This chapter therefore seeks to demonstrate whether insulin may inhibit the differentiation of myofibroblasts by altering the absolute and relative expression of the TGF- β isoforms in favour of a less pro-fibrotic (less TGF- β 1 and 2) or more anti-fibrotic (more TGF- β 3) combination. In addition, the issue of insulin and autocrine TGF- β 1 will be explored in relation to transient TGF- β 1 treatment and fetal myofibroblast differentiation.

Aims

- To determine whether insulin affects the transcription of the TGF- β 1 gene or the ratio of pro- to anti-fibrotic TGF- β isoforms in terms of their levels of mRNA.
- To demonstrate whether insulin affects the protein expression of TGF- β 1.
- To see whether insulin inhibits myofibroblast differentiation after *transient* exposure to TGF- β 1.
- To show whether insulin affects fetal myofibroblast differentiation.

4.2 Does insulin affect fibroblast mRNA expression of TGF- β 1?

4.2.1 Introduction

TGF- β 1 mRNA levels are elevated in keloid (Lee et al. 1999) and hypertrophic scar (Wang et al. 2000) fibroblasts compared to normal fibroblasts and are decreased in fetal fibroblasts (Martin et al, 1993). In other words, both extremes of scar tissue production appear to correlate with TGF- β 1 mRNA levels. Therefore, it is possible that insulin exerts its anti-scarring effects by depressing TGF- β 1 mRNA levels and it is the purpose of this section to see whether insulin treatment does indeed have this consequence.

However, changes in mRNA levels do not automatically correlate with the final protein expression levels so the results must be interpreted in the combination with protein quantification (section 4.4). The addition of exogenous TGF- β 1 to fibroblast cultures mimics the release of paracrine TGF- β 1 at the time of wounding which perhaps allows a more realistic assessment of the changes in autocrine TGF- β 1 mRNA levels that occur in the healing wound. This section will therefore test insulin's ability to affect TGF- β 1 mRNA levels following exogenous TGF- β 1 and no TGF- β 1 treatment.

4.2.2 Results

Normal scar fibroblasts were cultured in triplicates in 6 well plates at a density of 1×10^5 cells per well and harvested with twice weekly TRIZOL[®] for the duration of the three week time course (see section 2.8 for detailed description of method). On T0 the

media in the wells was changed to NGM or DTTM with and without bovine insulin (5µg/ml).

The extracted RNA samples were converted into cDNA and then subjected to multiplex RT PCR with TGF-β1 and GAPDH PCR primers (as detailed in section 2.6). GAPDH is a ubiquitously expressed housekeeping gene that reliably generates bands at 200 bps after PCR with specific primers. Although it shows a great deal of variation in levels of expression between different tissues, its expression by a single tissue remains constant and furthermore its expression is not known to be effected by insulin (Barber et al. 2005), see also appendix I.

The typical appearance of a multiplex PCR gel bands representing GAPDH and TGF-β1 is shown in figure 4.2.1. The ratio of TGF-β1 to GAPDH mRNA levels over time in NGM, DTTM or DTTM plus insulin is shown in figure 4.2.2. There are indications that TGF-β1 mRNA levels are consistently albeit slightly less at every time point in the insulin-treated fibroblasts compared to the untreated fibroblasts though not reaching but approaching statistical significance at T3 (P=0.163) and T10 (P=0.138). In addition, there is an indication of a decline in TGF-β1 mRNA levels over time that is particularly notable in the insulin-treated media but again fails to reach significance (P=0.128).

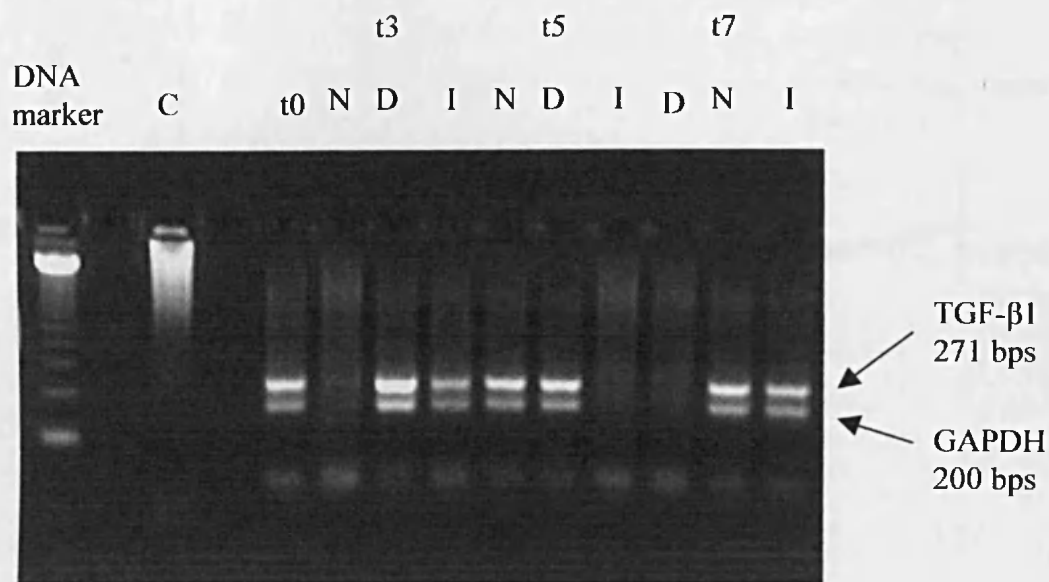


Fig. 4.2.1 Typical appearance of multiplex PCR gel showing GAPDH (200 base pairs) and TGF- β 1 (271 base pairs) primers. (C – control – DEPC water used in place of cDNA), t0 – day 0 (DTTM), (DTTM, N-NGM, D –DTTM, I – DTTM + insulin). This gel shows the ratio of mRNA expression of TGF- β 1 and GAPDH by a single cell strain in the three different media at the first three time points of a 17 day time course.

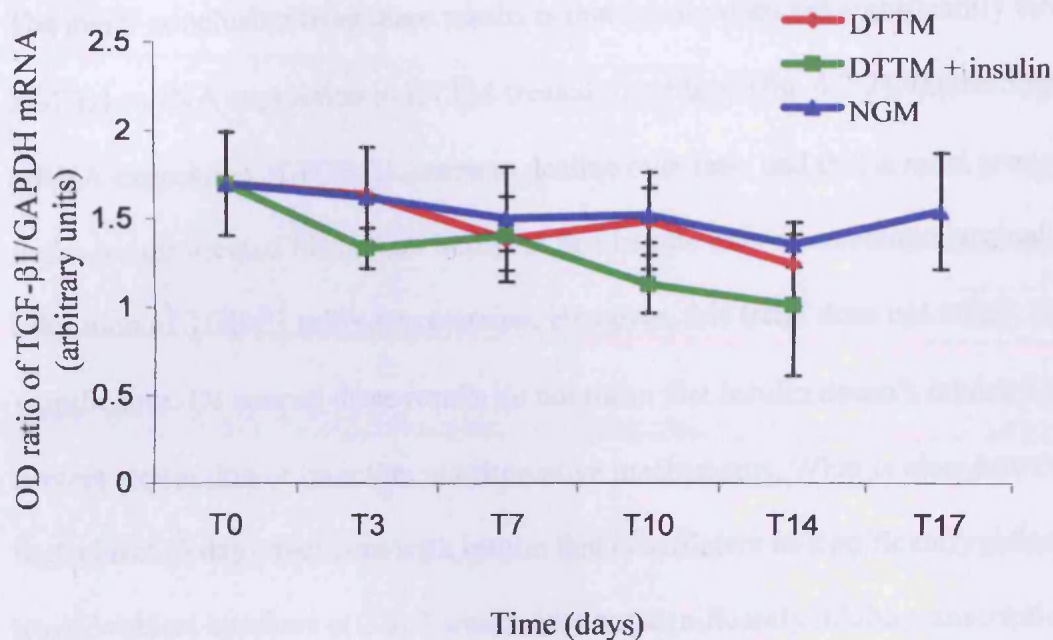


Fig. 4.2.2 OD ratio of TGF- β 1/GAPDH mRNA of normal scar fibroblasts over 17 day time course. Media: DTTM with and without insulin (5 μ g/ml) and NGM. N=3 cell lines. No significant difference between media at any time point using Student t-tests. Comparing T0 with T14 using t-tests: DTTM P=0.128, DTTM plus insulin P=0.128 and NGM P=0.137. At T3 using t-tests: DTTM plus insulin compared with DTTM P=0.163. At T10 using t-tests: treated compared with NGM P=0.138.

4.2.3 Discussion

The major conclusion from these results is that insulin does not significantly inhibit TGF- β 1 mRNA expression in DTTM-treated fibroblasts (fig. 4.2.2). Interestingly, the mRNA expression of TGF- β 1 seems to decline over time and this is most pronounced in the insulin-treated fibroblasts implying that insulin may exert some marginal inhibition of TGF- β 1 mRNA expression. However, this trend does not reach significance. Of course, these results do not mean that insulin doesn't inhibit TGF- β 1 protein expression or its action via alternative mechanisms. What is clear however is that a brief (3 day) treatment with insulin that is sufficient to significantly reduce myofibroblast numbers at 2 to 3 weeks does not significantly inhibit transcription of the TGF- β 1 gene. It also remains possible that insulin could act by altering the ratio of TGF- β isoform transcription levels.

4.3. Does insulin effect the mRNA expression of TGF- β 2 and 3?

4.3.1 Introduction

As far as the author is aware there are no published associations between insulin and the other TGF- β isoforms, 2 and 3. Nevertheless, their affirmed roles in scarring/anti-scarring mechanisms mean that, just as with TGF- β 1, their relationship with insulin must be explored. However, their investigation is more challenging than with TGF- β 1 as they tend to be expressed at much lower levels if at all. But with their baseline expression levels being so low, it seems more probable that in contrast with TGF- β 1, any significant increases in their expression will include increases in their mRNA levels, rather than just protein translation. If insulin does work by changing the relative expression of these two factors then the expected change would be a decrease in the expression of TGF- β 2 and/or increase in the expression of TGF- β 3. This section aims to see whether this hypothesis is met.

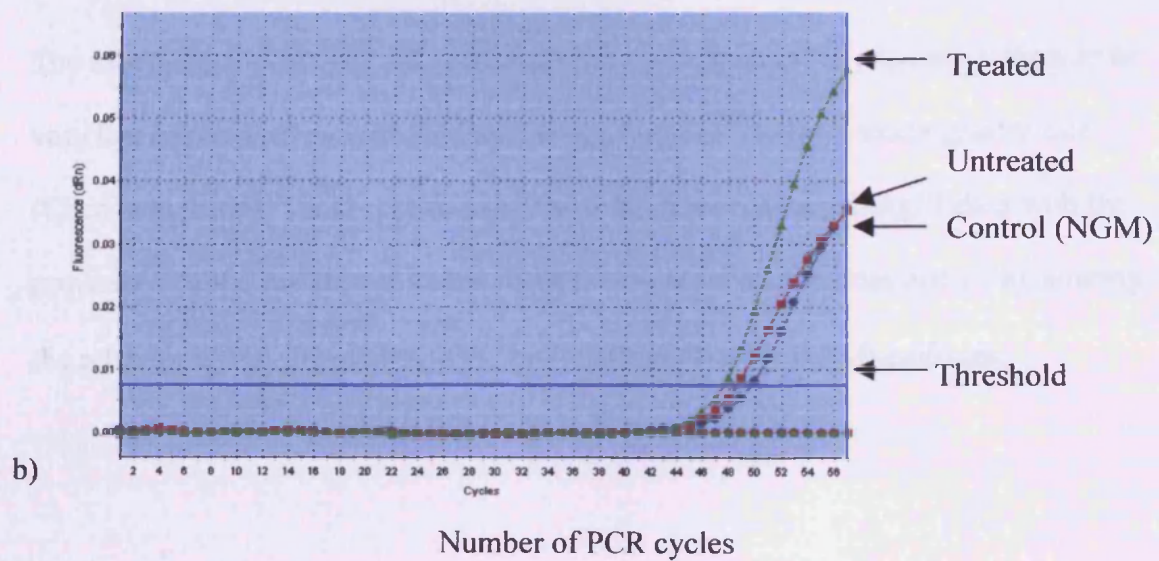
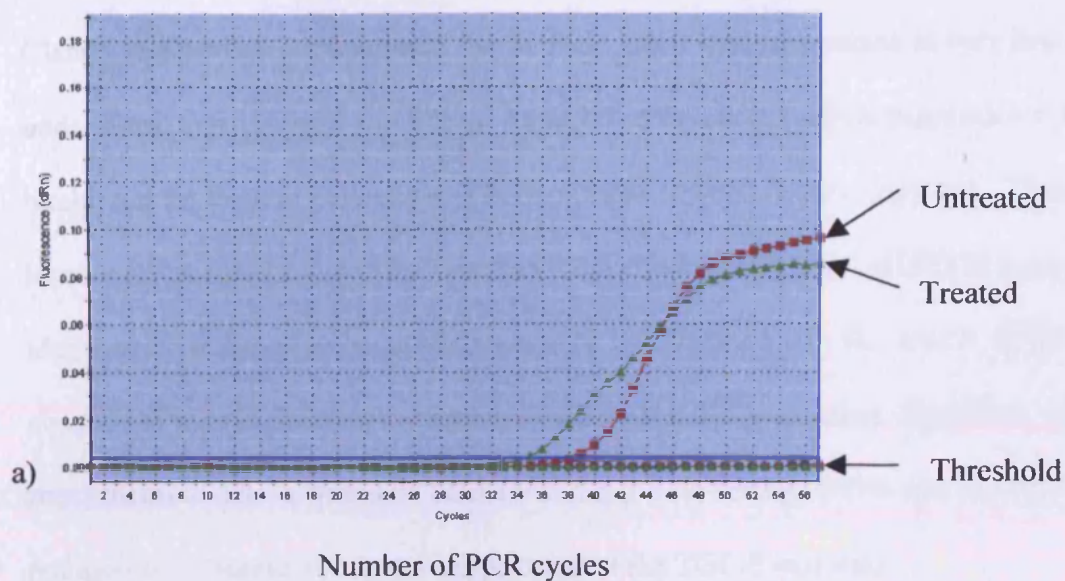
4.3.2 Results

Normal scar fibroblasts were cultured in triplicates in 6 well plates at a density of 1×10^5 cells per well and harvested with twice weekly TRIZOL[®] for the duration of the three week time course. On T0 the media in the wells was changed to NGM and DTTM with and without bovine insulin (5 μ g/ml). The extracted RNA samples were converted into cDNA and then subjected to multiplex RT PCR with TGF- β 2 or 3 and GAPDH PCR primers (see section 2.8).

The TGF- β 2 and 3 primers generated barely discernible bands that weren't suitable for multiplex PCR densitometric analysis (not shown). This indicates either that the mRNA levels of TGF- β 2 and 3 are very low or that the primers did not work (although this is unlikely as the same primers were used successfully by Rolfe et al., 2007b). To confirm these results the primers and samples were put through real time RT PCR (see figs. 4.3.1 a and b). This confirmed that the primers could be successfully used for PCR amplification, however both the TGF- β 2 and 3 genes were being expressed at very low levels and with no significant differences (see table 4.3.1) in expression between insulin-treated and untreated for either isoform. Interestingly this low level of expression seen in the DTTM is identical to that seen in NGM (Figs. 4.3.1 b).

Sample/primer	Average cycle number at threshold	+/- standard deviation	P number treated vs untreated
Untreated/TGF- β 2	37.1	3.31	
Insulin treated/TGF- β 2	33.97	2.28	0.295
Untreated/TGF- β 3	46.98	2.75	
Insulin treated/TGF- β 3	47.83	1.94	0.946

Table 4.3.1 Real time RT PCR of insulin treated and untreated normal scar fibroblasts cultured in DTTM (TGF- β 2 and TGF- β 3 primers). N=8.



Figs. 4.3.1 a and b. Representative real time multiplex RT-PCR plots for a) TGF- β 2 and b) TGF- β 3. N=8. The point at which the plots cross the blue threshold line represents the number of PCR cycles at which the TGF- β 2 or 3 DNA becomes detectable. This is at approximately 40 cycles for both genes indicating their very low levels of expression.

4.3.3 Discussion

Clearly, the mRNA expression of the TGF- β 2 and 3 isoforms occurs at very low levels under these experimental conditions. At such low levels of mRNA expression it is likely that the protein expression of both of these isoforms is also very low. These low levels are not simply due to the less than ideal culture conditions of DTTM since identical expression levels are also seen in NGM. Furthermore, this media, in the absence of insulin, induces increased myofibroblast differentiation. Therefore, insulin's mechanism of action would be unlikely to depend on these proteins and insulin does not appear to change the relative expression of the TGF- β isoforms.

The expression of the TGF- β 2 and 3 isoforms in adult skin is in any case known to be very low in comparison with fetal skin where they seem to have much greater role (Chen et al. 2005). These results are perhaps therefore not surprising. Taken with the previous TGF- β 1 results, the overall impression is that insulin does not act by altering the relative mRNA expression of the pro- and anti-fibrotic TGF- β isoforms.

4.4 Does insulin effect the protein expression of TGF- β 1?

4.4.1 Introduction

Previous work in this chapter suggests that insulin does not alter the relative mRNA expression of the TGF- β isoforms and specifically that it doesn't significantly decrease the transcription levels of the TGF- β 1 gene. However, it cannot be concluded from this that insulin does not act by reducing the levels of TGF- β 1 as it may act on protein translation rather than mRNA transcription and this requires investigation.

Upregulation of TGF- β 1 protein expression is induced by a variety of molecules including steroids, retinoids, EGF, nerve growth factor, activators of lymphocytes, vitamin D3, IL 1 and by TGF- β 1 itself (Kim et al. 2005). Its synthesis is also well documented to be inhibited by a range of molecules including EGF, fibroblast growth factor (FGF), dexamethasone, calcium, retinoids and follicle stimulating hormone (Kim et al. 2005). It is therefore quite feasible that insulin too can down regulate the protein expression of TGF- β 1. If so, this would be a convincing mechanism of insulin's antifibrotic action. The aim of this experiment is to test this possibility.

4.4.2 Results

Three normal scar cell strains were incubated in NGM, DTTM and DTTM plus insulin for 14 days and then further incubated in serum free media for 24 hours. This conditioned media was then subjected to sandwich linked ELISA using anti- TGF- β 1 antibodies as per the method outlined in section 2.7.

The results (fig. 4.4.1) demonstrate that for each cell strain there is no significant difference in the levels of TGF- β secreted by fibroblasts in DTTM and DTTM plus insulin. In contrast the fibroblasts pre-incubated in NGM show significantly higher TGF- β 1 secretion than the fibroblasts in the other test media ($p < 0.05$).

4.4.3 Discussion

These results (fig. 4.4.1) strongly suggest that insulin does not affect the protein expression of TGF- β 1. Taken with the previous results relating to mRNA expression it would appear that insulin doesn't affect TGF- β 1 expression at all. The fibroblasts cultured in NGM show much higher levels of TGF- β 1 secretion but this is expected as NGM contains many more growth factors and proteins. These results suggest that insulin's mechanism of action is not via the expression or secretion of TGF- β 1 and so if this growth factor is involved then it must be in terms of its action or activation.

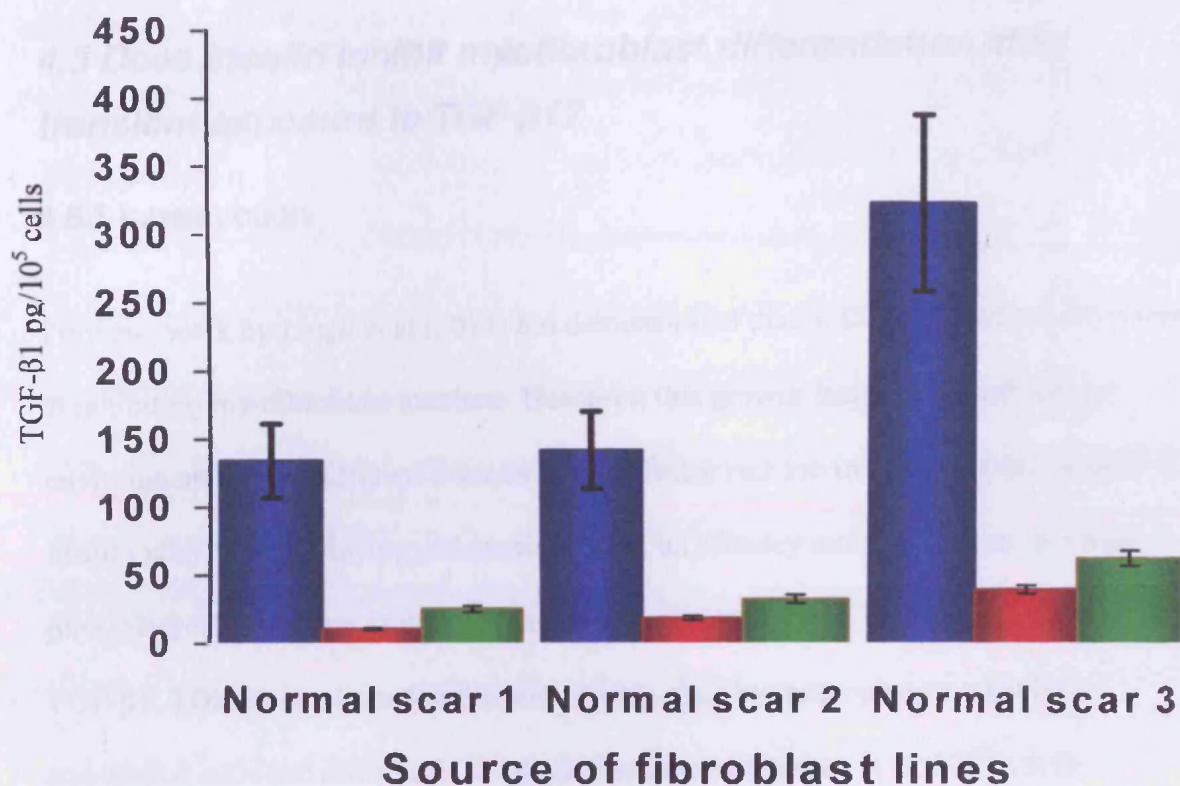


Fig. 4.4.1. Sandwich linked ELISA using anti- TGF-β1 antibodies of media taken from fibroblast cultures pre-incubated in NGM, DTTM and DTTM plus insulin for 14 days and then in serum free media for 24 hours. N = 3. No significant difference in the level of TGF-β secretion between fibroblasts in DTTM and DTTM plus insulin. Fibroblasts pre-incubated in NGM show significantly higher TGF-β1 secretion than the fibroblasts in the other test media (Student t-test, $P < 0.05$).

4.5 Does insulin inhibit myofibroblast differentiation after transient exposure to TGF- β 1?

4.5.1 Introduction

Previous work by Linge et al (2004) has demonstrated that in DTTM insulin is effective at inhibiting myofibroblast numbers. However, this growth factor deprived culture environment is very different from the growth factor rich environment of the wound. If insulin is to be a convincing antiscarring agent, its efficacy must be proven in a more physiologically relevant culture environment, one with growth factors in particular TGF- β 1. TGF- β 1 is released at the time of wounding by macrophages, platelets, endothelial cells and other cells in a paracrine fashion (Beanes et al. 2003). It is predominantly the TGF- β 1 that stimulates differentiation of fibroblasts into myofibroblasts and scar formation. Grotendorst et al. (2004) showed that as little as 30 minutes exposure to exogenous TGF- β 1 is enough to stimulate 50% of the maximal DNA synthesis response in fibroblasts and this effect persists well after its removal from the culture medium. It also stimulates the fibroblasts to produce their own, autocrine, TGF- β 1 (Chin et al. 2004) as well as other cytokines that can mediate its longer-term effects, such as connective tissue growth factor (CTGF) and IGF-II, without its continuous presence being necessary (Grotendorst et al, 2004). The release of paracrine TGF- β 1 is short-lived whereas the expression of the autocrine TGF- β 1 is more sustained (O'Kane et al. 1997).

However, as section 3.4 demonstrates, neither acute nor chronic treatment with insulin prevents the initial induction of myofibroblast differentiation by exogenous active (paracrine) TGF- β 1. However, both of these experiments assessed the proportion of myofibroblasts after only 4 days TGF- β treatment. It is quite possible that insulin could affect myofibroblast numbers at a later time point such as 14 days after the TGF- β treatment has ceased. The reasoning behind this is the finding by Mackie (2004) that using the incisional wound healing model in Balbr mice that whilst insulin treatment did not significantly alter the initial peak of myofibroblasts seen approximately 7 days after wounding, a significant reduction to barely detectable levels was seen at 14 days (Mackie 2004). In contrast, the placebo treated wounds maintained a distinct shoulder of higher than normal myofibroblast numbers for 14 to 28 days. This scenario reflects an effect of insulin on the longevity of myofibroblasts or their continued promotion by autocrine TGF- β 1 stimulated by the original burst of paracrine active TGF- β .

An alternative experiment was formulated in an attempt to mimic the TGF- β 1 stimulating events that go on within an actual wound. This experiment employs a different test medium: 0.4% FCS. The advantage of this medium is that it contains the minimal amount of FCS to maintain cells in a quiescent state but at the same time prevent apoptosis (personal communication, C. Linge, RAFT). This is particularly useful for experiments involving prolonged time courses where proliferation would make it difficult to interpret whether differences in myofibroblast numbers are due to inhibition of differentiation or the effects of differential proliferation. Although this medium does contain active insulin and TGF- β 1, their concentrations are low enough to

be negligible compared to the amounts added in the relevant experiments. Exogenous TGF- β is then added to these cells for 3 days only to mimic the presence in the wound of active TGF- β released from platelets and other cells. The differentiation into myofibroblasts is then followed over a long time course.

4.5.2 Results

Normal scar fibroblasts cultured on triplicate coverslips in 0.4% FCS were either maintained in that medium or exposed to exogenous TGF- β 1 (2ng/ml), with and without a once only dose of insulin (5 μ g/ml), both of which were removed after the first three days of a longer subsequent time course (up to 17 days). After the initial three days media was refreshed regularly but no further TGF- β 1 or insulin was added. At each time point (every 3-4 days) the coverslips were harvested, immunostained and the proportion of myofibroblasts recorded.

The addition of TGF- β 1 to the media, although only present for the first three days only, is associated with an approximately three-fold increase in the proportion of myofibroblasts compared to the cultures in the untreated media (fig. 4.5.1). Even after 7 days when the exogenous TGF- β 1 has long been removed, the proportion of myofibroblasts in the TGF- β 1-only treated cultures continues to increase reaching a plateau (approximately 27%) after day 10. In contrast, the proportion of myofibroblasts in the insulin and TGF- β 1 treated cells peaks at 7 to 10 days and declines thereafter such that by day 17 (approximately 17%) it is significantly less than TGF- β -only treated cells although it is still higher than in the untreated cells.

The experiment performed in section 4.2 was also repeated with cells that were pulsed (treated for 3 days only) with TGF- β 1 in an attempt to mimic the early release of paracrine TGF- β in the wound. This was done in the presence and absence of insulin and the cells were harvested for Multiplex PCR at day 0, day 3 and every 3 or 4 days thereafter until day 17. These results (fig. 4.5.2) indicate that as in fig. 4.2.2, insulin does not affect the mRNA expression of TGF- β 1 in the presence or absence of pulsed TGF- β 1 treatment.

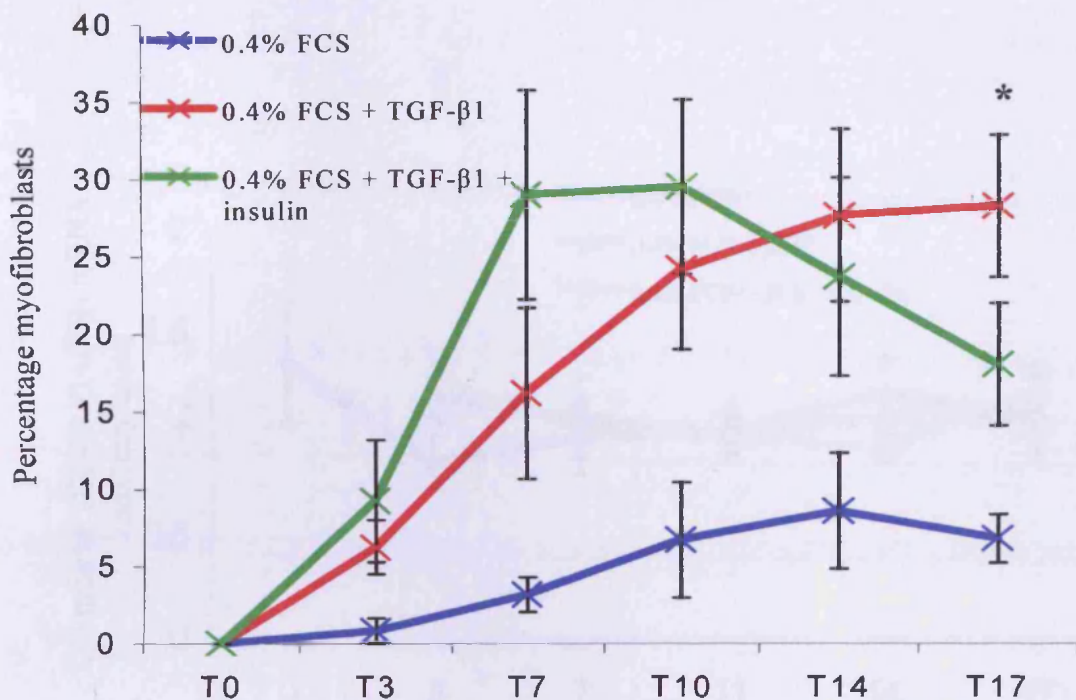


Figure 4.5.1 Normal scar fibroblasts cultured over 17 days in 0.4% FCS. Initial 3 day treatment with exogenous recombinant human TGF- β 1 (2ng/ml) with and without insulin (5 μ g/ml). All cells cultured in 0.4% FCS only beyond day 3. Control: 0.4% FCS only. N=4. Significant difference between both treatment groups and the control, Mann Whitney Rank tests: T7 - $P < 0.05$, T10 - $P < 0.05$, T14 $P < 0.01$, T17 $P < 0.049$. Significant difference between treatment groups at (*) day 17 (T-test $P < 0.005$).

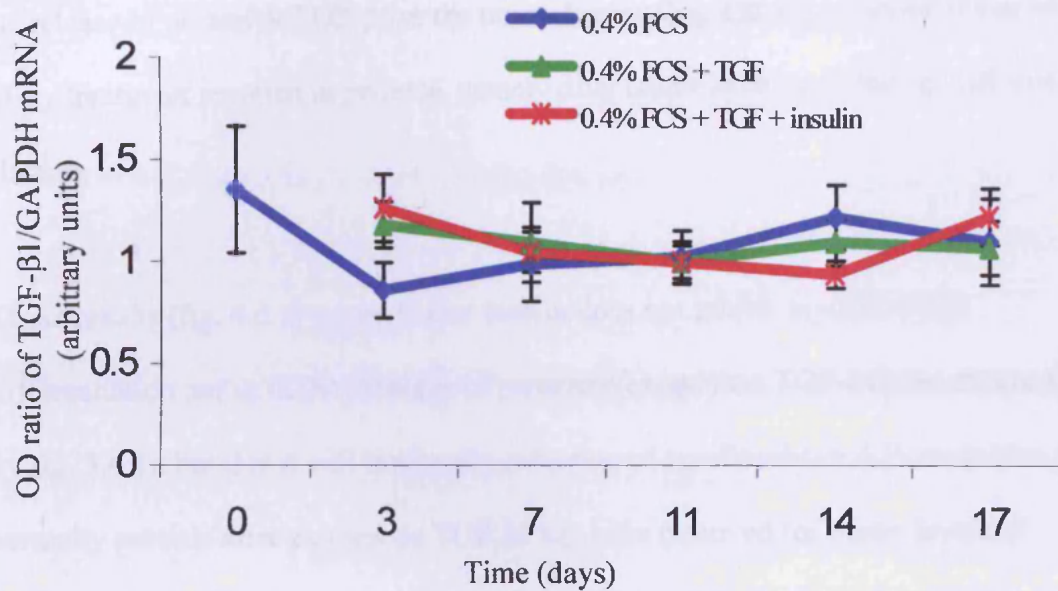


Fig. 4.5.2 Multiplex PCR quantification of TGF- β 1 gene expression. Normal scar fibroblasts cultured over 17 days in 0.4% FCS. Initial 3 day treatment with exogenous recombinant human TGF- β 1 (2ng/ml) with and without insulin (5 μ g/ml). All cells cultured in 0.4% FCS only beyond day 3. Control: 0.4% FCS only. N = 4 cell strains. No significant difference between treatment groups and control.

4.5.3 Discussion

The addition of once only doses of TGF- β 1 and insulin has been employed to mimic the release of paracrine TGF- β 1 at the time of wounding and also recreate the most likely treatment regimen in patients, namely drug administration at the time of wound closure.

These results (fig. 4.5.1) suggests that insulin does not inhibit myofibroblast differentiation per se in the presence of paracrine/exogenous TGF- β 1 (also demonstrated by fig. 3.4.1) but that it will inhibit the induction of myofibroblast differentiation that normally persists after exogenous TGF- β 1 has been removed (or tissue levels of paracrine TGF- β decline as normally happens in the wound). For this to happen insulin must directly or indirectly inhibit the mediator(s) of paracrine TGF- β 1's longer-term effects, such as the induction/activation or action of autocrine TGF- β 1. However, the results illustrated in figure 4.5.2 that these effects of insulin are not due to the inhibition of the mRNA expression of autocrine TGF- β 1 which makes mechanisms involving the activation of TGF- β more plausible than mechanisms involving its expression. These ideas will be explored in chapter 5.

4.6 Does insulin affect fetal myofibroblast differentiation?

4.6.1 Introduction

The ability of the fetal skin to heal full thickness wounds with little or no scarring has been well documented (Ferguson et al. 2004) (see section 1.8). There is evidence that the relative expression of TGF- β isoforms, specifically an increased ratio of TGF- β 3 compared to TGF- β 1 and 2, is the underlying mechanism (Shah et al. 1995).

Nevertheless, work by Rolfe and co-workers has indicated a potential role for a differential cellular response to TGF- β 1. Rolfe et al. (2007a) demonstrated that early gestational age fetal (non-scarring) fibroblasts differentiated into myofibroblasts on stimulation with TGF- β much more transiently than their more developmentally mature counterparts. This group went on to show that this correlated with an inability of TGF- β 1 to stimulate transcription of its own gene in these cells, whereas it significantly stimulated TGF- β 1 gene transcription and release of autocrine TGF- β in postnatal cells (Rolfe et al. 2007b). It is possible that insulin inhibits adult scarring via effects on autocrine TGF- β or its action. The purpose of this experiment is to see whether the response of fetal fibroblasts to insulin is different to that of adult fibroblasts and thus indicate whether this potential mechanism could be involved.

4.6.2 Results

The previous experiment (see section 4.5) was repeated using human fetal dermal fibroblasts from an early gestation (EGA 12) aborted fetus in an attempt at reproducing

Rolfe et al's findings under the same experimental conditions as those used in section 4.5 that is, 0.4% FCS, rather than the severe quiescence-inducing conditions of their original study. Unfortunately, owing to the difficulty of obtaining and subsequently culturing fetal cells, it was only possible to perform this experiment with one cell line in triplicate. The fetal fibroblasts were cultured on coverslips in 0.4% FCS and were either maintained in this medium only or transiently exposed to exogenous TGF- β 1 (2ng/ml) with and without insulin (5 μ g/ml). The media was replaced with basic media only after 3 days. The coverslips were harvested at intervals over a 17 day time course and stained with FITC-conjugated α -SMA antibodies and propidium iodide and the proportion of myofibroblasts determined.

The results (see fig. 4.6.1) show a marked increase in myofibroblast numbers in the fetal cell cultures between days 0 and 10 similar to the response in the adult cells (fig. 4.5.1). In contrast however, both treatment groups demonstrated a sharp peak (fig. 4.6.1) rather than the prolonged presence of myofibroblasts for up to 17 days in the way that the TGF- β 1 treated adult cells did (fig. 4.5.1).

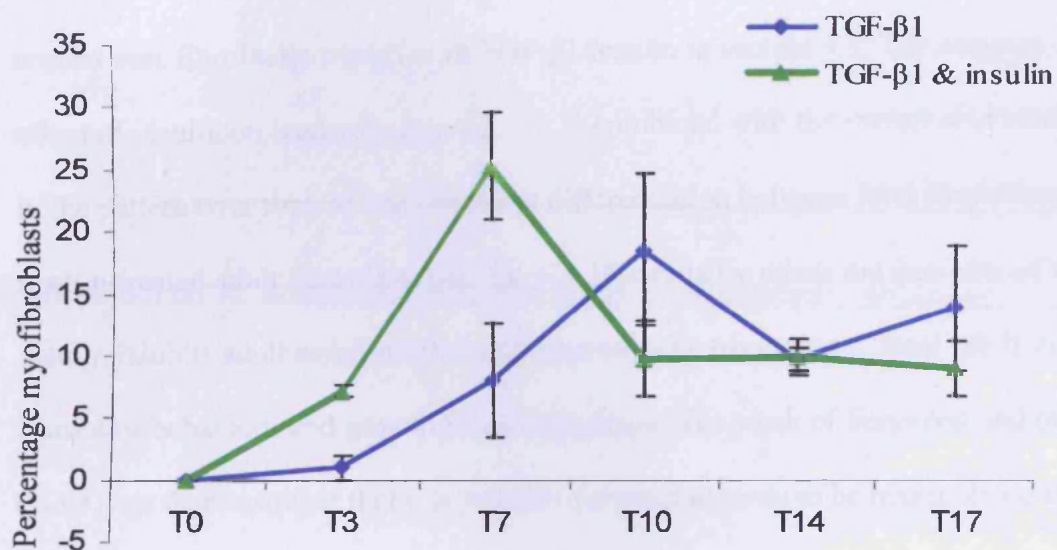


Fig. 4.6.1 Fetal dermal fibroblasts cultured over 17 days in 0.4% FCS. Exogenous recombinant human TGF- β 1 (2ng/ml) with and without insulin (5 μ g/ml) was present for the first 3 days only and cells were cultured in 0.4% FCS only thereafter. Control: 0.4% FCS only. N=1 cell strain, repeated three times. N number not sufficient for statistical analysis.

4.6.3 Discussion

Both the insulin treated and untreated fetal fibroblasts (see fig. 4.6.1) display a similar pattern of change in myofibroblast numbers with a peak at 7 to 10 days followed by a decline and then reaching a plateau. This resembles the pattern exhibited by the adult normal scar fibroblasts treated with TGF- β 1/insulin in section 4.5. The absence of effect of insulin on fetal cells (see fig. 4.6.1) combined with the remarkable similarity in the pattern over time of myofibroblast differentiation between fetal fibroblasts and insulin-treated adult fibroblasts (see fig. 4.5.1) inevitably raises the question of whether insulin inhibits adult myofibroblast differentiation by triggering a 'fetal' shift in adult fibroblast behaviour and growth factor expression. The work of Ferguson and others (2004) has demonstrated that it is possible for adult wounds to be manipulated through changes in the TGF- β isoform profile into behaving in a more fetal manner with improved or no scarring. Nevertheless, the work presented in sections 4.2 and 4.3 suggest that this is not how insulin works.

4.7 Discussion and Conclusion

The results from sections 4.2, 4.4 and 4.5 suggest that there is no significant effect of insulin treatment on TGF- β 1 mRNA levels or protein expression. The mRNA levels of TGF- β 1 are unchanged, although there was a possible trend towards a decline in TGF- β 1 expression by fibroblasts after long-term (14-17 days) treatment with insulin (see fig. 4.2.2) although this was not significant. This may represent inhibition of basal autocrine TGF- β 1 expression by fibroblasts. However this trend was not found over a similar time course in fibroblasts exposed to short-term (3 days) insulin treatment (see fig. 4.5.2). This is a powerful stimulus to autocrine TGF- β 1 production by the fibroblasts and therefore if insulin does inhibit autocrine TGF- β 1 action following wounding or exogenous TGF- β 1 treatment, then it must do so by a means other than the mRNA expression of TGF- β 1. The findings from the TGF- β ELISA (fig. 4.4.1) suggest this impression can be extended to include the secretion of the TGF- β 1 protein. Thus, it can be reasonably concluded that insulin's mechanism of action is not the inhibition of the mRNA or protein expression of TGF- β 1.

Additionally, the finding that TGF- β 2 and 3 are expressed at only very low levels (see section 4.3), at least in terms of mRNA within these in vitro environments, strongly suggest that the myofibroblast inhibition we see within these cultures is not due to a change in the TGF- β isoform ratio in favour of a anti-fibrotic balance, that is, an increase in TGF- β 3 and a decrease in TGF- β 1 and/or 2. This finding that the mRNA expression of the TGF- β isoforms 2 and 3 was at barely detectable levels, led to the

assumption that they do not play an important role in insulin's mechanism of action. However, although it is unlikely that at these low levels TGF- β 2 and 3 do play a role, it is possible that it is incorrect to extrapolate a low mRNA expression to meaning a low bioactivity of the protein products. There are genes expressed in only very low mRNA levels but nevertheless have profound affects on cellular behaviour and this is particularly true of growth factors. Furthermore these experiments are restricted to in vitro cultures but it could be that in the in vivo environment relative TGF- β isoform expression does play a much greater role in insulin's ability to inhibit scarring. Nevertheless, it is important to establish whether the relative expression of the TGF- β isoform do have a role in insulin's mechanism of action. This is because the main potential anti-scarring therapies (Ferguson et al. 2004) currently in development are based on an alteration of the relative levels of the TGF- β isoforms. If insulin works by a different mechanism, then the possibility of combination anti-scarring therapies involving insulin and TGF- β isoform effecting agents is opened up. Experience with combination therapies in other medical disciplines shows the great efficacy of this approach, for example, anti-hypertensives, cancer chemotherapies and antibiotics all show greater efficacies when used as combinations of agents that have complementary, rather than competing mechanisms of action.

The results of 4.2-5 are not contradicted by the current state of knowledge about insulin which as far as the author is aware does not include any reports of insulin influencing the relative expression of the TGF- β isoforms. However, insulin is a hormone with pronounced effects on the expression and distribution of a wide range of receptors

involved in anabolic activities and even immunomodulatory functions (Cohen 2006). Insulin modulates many of its metabolic functions via the phosphorylating activity of its tyrosine kinase receptor but induces its other actions such as glycogen and protein synthesis, mitogenesis, inhibition of apoptosis and regulation of gene transcription via phosphatidylinositol 3-kinase (PI 3-kinase) activity (Siddle et al. 2001). Insulin has even been shown to directly affect the expression of other growth factors such as VEGF (Van Obberghen et al. 2001) and to stimulate the expression (Oka et al. 1984) and redistribution of receptors such as the IGF-II receptor (Oka et al. 1985; Corvera et al. 1988). Thus it is not implausible that insulin might affect the expression/action additional growth factors such as TGF- β .

However, the relative levels and activity of the TGF- β isoforms in scarring and non-scarring wounds can be altered in more ways than by mRNA expression and protein translation alone. For example, in the fetal wound there are far fewer degranulating platelets to release the initial dose of TGF- β 1 (Shaw 2000) followed by a weaker inflammatory response, which results in less infiltration of TGF β 1-producing inflammatory cells (Cowin et al. 1998). These dual factors combine to produce much lower levels of TGF- β 1 but in addition to the relative levels of the TGF- β isoforms, they can be activated to different degrees as well, for example, Soo et al revealed that fetal wounds contain more TGF- β modulator fibromodulin, which inhibits TGF- β activation than adult wounds (Soo et al. 2000). The expression of TGF- β receptors may also vary to have similar effects for example Hsuo et al demonstrated that fibroblasts from the early gestation fetus express lower levels of TGF- β receptor II (Hsu et al.

2001). Furthermore, the signalling of these receptors can also be modulated, for example, by differential expression of receptor tyrosine kinases between fetal and adult fibroblasts, seen in rats (Chin et al. 2001).

This chapter has also demonstrated that insulin can significantly inhibit myofibroblast differentiation in fibroblast cultures even after transient exposure to the powerful myofibroblast inducer, TGF- β 1 (see fig. 4.5.1). This finding gives important fillip to insulin's antiscarring credentials but also hints at its mechanism of action. Insulin cannot overcome the myofibroblast inducing effects of exogenous (or paracrine) active TGF- β 1 but does significantly inhibit TGF- β 1's longer-term effects that occur days after its removal. Therefore, it can be concluded that insulin does not inhibit the initial action of activated TGF- β 1 but may inhibit the actions of secondary mediators such as autocrine TGF- β 1 itself or CTGF (connective tissue growth factor).

Under the conditions used for the experiments in this thesis, the 'non-scarring' phenotype fetal cells (insulin treated and untreated) (fig. 4.6.1) only have a transient myofibroblast differentiation response to TGF- β 1 which is very reminiscent of that seen in the developmentally mature normal scar cells after treatment with insulin (fig. 4.5.1). In addition, insulin has no effect on the numbers of fetal myofibroblasts at the late time points (days 14 and 17). This raises the possibility that insulin inhibits adult scarring by influencing the temporal pattern of myofibroblast differentiation to follow a more 'fetal' pattern. However, the results from section 4.2 – 4.4 suggest this is not due to an insulin-induced change in the relative expression of the TGF- β isoforms. Alternatively

we know that early gestation fetal fibroblasts do not respond to exogenous (paracrine) TGF- β 1 with production of autocrine TGF- β 1 as adult fibroblasts do (Rolfe et al., 2007b). In this scenario, if insulin's mechanism were to involve autocrine TGF- β 1, then an absence of effect of insulin on fetal cells as has occurred here (fig. 4.6.1), would be expected. Indeed, Martin et al found that both the gene expression and protein production of TGF- β 1 were much more transitory in fetal wounds compared to adult wounds and that the gene expression and protein production of TGF- β 1 was slower and more sustained in the adult wounds (Martin et al. 1993).

Thus although the results presented here do not indicate that insulin changes the relative expression of the different TGF- β isoforms, it is possible that insulin exerts effects through changes in the relative activity of TGF- β 1. This is a question that would bear more investigation. One of the main regulators of availability of bioactive TGF- β is its activation since it is normally secreted in a latent form. The influence of TGF- β 1 can therefore be mitigated not just by decreasing its mRNA or protein expression but by also inhibiting its activation. The next chapter will explore this issue.

4.7.2 Conclusions

1. Insulin does not significantly change the mRNA (see figs 4.2.2 and 4.2.3) or protein expression (see fig. 4.4.1) of endogenous TGF- β 1 by fibroblasts in these experimental models.
2. The mRNA expression of TGF- β 2 and 3 is very low in these experimental models (see section 4.3) and therefore insulin's mechanism of action is unlikely to be due to modification of the ratio of expression of the three TGF- β isoforms.
3. Insulin does inhibit the prolonged presence of myofibroblasts (after 7 to 10 days) following transient TGF β -1 treatment (see fig. 4.5.1). This may be indicative of an effect on autocrine TFG β 1.
4. The pattern of the change in myofibroblast numbers over time in insulin and TGF β -1 treated adult fibroblast cultures (fig. 4.5.1) is very similar to the pattern observed in TGF β -1 treated (with and without insulin) fetal cells (fig. 4.6.1), that is, decreased numbers are seen after day 7. Insulin appears to have little effect on fetal non-scarring fibroblasts.

Chapter 5

**Does insulin exert its inhibitory
effect on myofibroblast
differentiation via activation of
autocrine transforming growth
factor beta?**

5.1 Introduction

The previous chapter demonstrated that insulin does not appear to inhibit the levels of expression of TGF- β 1 nor significantly alter the ratio of TGF- β isoforms. An alternative hypothesis is that insulin still might act through its effects on the TGF- β system either by inhibiting the activation of latent autocrine TGF- β or the expression of its activating receptors. The aim of this chapter therefore is to specifically examine if insulin can affect the mechanisms of activation of autocrine TGF- β 1. Section 3.4 demonstrates that insulin does not inhibit myofibroblast differentiation in the presence of exogenous activated TGF- β 1. In the wound the early peak of TGF- β 1 is mainly of non-fibroblastic origin being either released from platelets or synthesized by the cells of the immune infiltrate. This “paracrine” TGF- β is either released active or is rapidly activated by enzymatic action within the early wound environment. The TGF- β present later in wound healing is thought mainly to be autocrine in origin. Autocrine TGF- β is secreted in an inactive form and so the activation of this latent TGF- β 1 will be investigated here. The activation of the latent complex of TGF- β is still not fully understood. However, there are two known main mechanisms by which the small latent complex (SLC – a dimer of mature TGF- β 1 [25 kDa and the latency associated peptide [40 kDa]) is activated. First is proteolysis by a variety of proteases such as plasmin, metalloproteinases, aspartic proteases, cysteine proteases, and serine proteases (Annes et al. 2004). Interaction with the IGF-II receptor causes activation of the SLC in this way (Ghosh et al. 2003). Integrins may also activate the SLC this way for example α V β 8 (an integrin expressed by normal epithelial and neuronal cells) activates the SLC

via an MT1-MMP-dependent pathway (Mu et al. 2002). The result of the proteolysis is release of active TGF- β 1 from the SLC. The second mechanism is a nonproteolytic conformational change of the LAP that allows activation of the SLC (McMahon et al. 1996). Thrombospondin-1 and α V β 6 appear to be involved in this process by interaction with N-terminus and C-terminus respectively (Annes et al. 2004; Daniel et al. 2004). However, α V β 6 expression is restricted to epithelial cells (Breuss et al., 1993). In fibroblasts α V β 5 has been shown to directly activate the SLC (Asano et al., 2005).

These different mechanisms of activating TGF- β 1 may all have the same final common pathway, namely disruption of the structure of the small latent complex resulting in decreased affinity between TGF- β 1 and its associated peptides and its release from the complex (Miyazono et al. 1991).

5.1.1 The IGF-II receptor

Evidence that the IGF-II receptor and the M-6-P receptor are one and the same protein came from Kiess et al in 1988 who found that antisera behaved identically towards the IGF-II receptor and M6P receptor in ligand blocking and immuno-precipitation assays (Kiess et al. 1988). These experiments came after the finding that there is 80% deduced amino acid sequence homology between the bovine cation independent M-6-P and the human IGF-II receptor (Morgan et al. 1987). There are in fact two M-6-P receptors. One is the 300kD glycoprotein, which binds M-6-P-containing proteins in the absence of divalent cations (Sahagian et al. 1981) and the other is a 46kDa membrane

glycoprotein, which also contains an M-6-P recognition site but whose function is unclear (Hoflack et al. 1985; von Figura K 1986). Interestingly the 300kDa M-6-P receptor binds IGF-II with significantly higher affinity than IGF-I but does not bind insulin (Kasuga et al. 1981; Massague et al. 1981). The surface expression of M-6-P receptors is increased by both the insulin-like growth factors and by a phorbol ester (Damke et al. 1992). The mechanism by which IGF-I and IGF-II induce the redistribution of M-6-P receptors is unclear but it is likely that IGF-I achieved this through the activation of the intrinsic tyrosine kinase activity of the IGF-I receptor (Ullrich et al. 1990) and IGF-II causes coupling of the M-6-P receptor to G_i II proteins (Murayama et al. 1990). In human fibroblasts the receptor redistribution induced by IGF-II does not depend on protein kinase C activation (Braulke et al. 1990), nor is it accompanied by changes in the formation of inositol triphosphate or cyclic ANP (Damke et al. 1992). Furthermore this redistribution is rapid and independent of protein synthesis (Damke et al. 1992). If insulin can interact with the M-6-P receptor itself to cause redistribution in this way then this gives a neat mechanism for explaining insulin's inhibition of the activation of autocrine TGF- β . Interestingly insulin has been reported to rapidly increase the binding affinity of the M-6-P receptor for IGF-II but not their number in rat adipocytes (Oppenheimer et al., 1983). If this effect is specific for the affinity of IGF-II and not latent forms of TGF- β then this may explain a mechanism of action of insulin.

Aims:

- To compare the effect on myofibroblast differentiation of insulin and M-6-P (a known blocker of latent TGF- β activation) to see whether insulin may inhibit myofibroblast differentiation in a similar way as M-6-P, that is via the M-6-P/IGF-II receptor.
- To determine whether insulin affects the activation of latent TGF- β in fibroblast cultures using immunostaining, Western blotting and bioassays.
- To see whether insulin affects the numbers of fibroblasts with detectable α V β 5 immunostaining.
- To demonstrate whether insulin affects the levels of the IGF-II receptor in terms of immunostaining, mRNA and protein expression.

5.2 The interaction between insulin, mannose-6-phosphate and TGF- β 1 and their effects on myofibroblast differentiation.

5.2.1 Introduction

The study of the activation of autocrine TGF- β 1 is aided by the use of mannose 6-phosphate (M6P), a known inhibitor of autocrine TGF- β 1 activation (Yang et al. 2000). M-6-P binds the IGF-II receptor and competitively inhibits the binding and subsequent activation of autocrine (latent) TGF- β 1. The insulin-like growth factor II (IGF-II) receptor, also known as the M-6-P receptor, is a 300kD cation-independent receptor. Cell transfection experiments have demonstrated that the receptor mediates the transport of newly synthesised acid hydrolases containing mannose 6-phosphate to lysosomes (Tarrago et al., 1999). The receptor mediates a trans-membrane signalling event in response to binding of IGF-II (Tarrago et al. 1999). It also binds a range of other extracellular ligands at the cell surface: plasminogen, urokinase-type plasminogen activator receptor (uPAR) and latent transforming growth factor beta; all involved in the regulation of cell growth and motility (Olson et al. 2004). This ability of the M6P receptor to interact with so many proteins is due to the receptor's large (approximately 2270 amino acids) extracytoplasmic region (Olson et al. 2004). The receptor's ligands include those that contain mannose 6-phosphates such as latent transforming growth factor-beta and ligands that don't contain mannose 6-phosphate including insulin-like growth factor II (Dahms et al. 2002). Importantly for this research, the M-6-P receptor enables the activation of TGF- β (Ghahary et al. 2000) and decreases serum levels of the mitogen IGF-II (Ludwig et al. 1996). Because the M-6-P receptor binds the simple

sugar mannose 6-phosphate and latent TGF- β binds to this receptor through its associated M-6-P groups, this means that addition of M-6-P to the cell culture medium competitively inhibits the binding of latent TGF- β to this receptor and thus allow us to study the effects of blocking the activation of TGF- β with and without the presence of insulin.

If insulin does indeed work by inhibiting the activation of autocrine TGF- β then we should expect insulin and mannose-6-phosphate to inhibit myofibroblast differentiation to similar degrees, the effects of the two added together should not be additive and with no antagonism of the other's effect. This hypothesis will be tested in following experiments.

5.2.2 Results

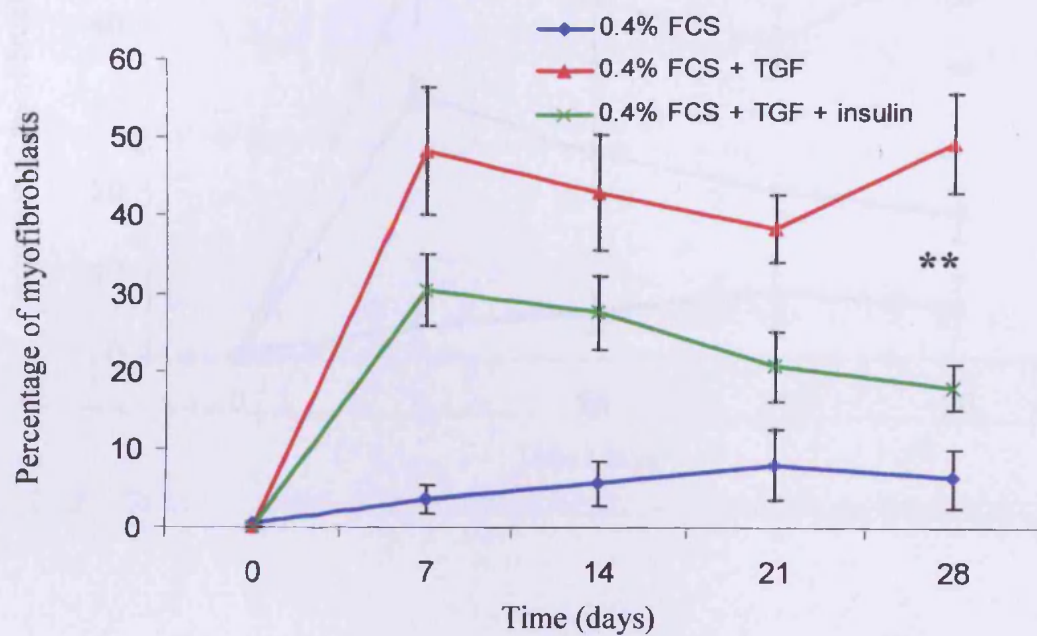
In this experiment normal scar fibroblasts were cultured on coverslips over a 28-day time course. The media used was 0.4% FCS; a fifth of the cells were cultured solely in this media; the rest of the cells were exposed to different media treatments as follows: A quarter were treated for the first three days only with TGF- β alone (2ng/ml) whereas a further quarter were treated with TGF- β 1 plus insulin for the first three days only and thereafter with 0.4% FCS media alone. Another quarter were treated with TGF- β 1 and insulin for the first three days followed by continuous mannose 6-phosphate treatment at a dose of 100 μ M and lastly, the final quarter, were treated with TGF- β 1 only for the first three days followed by continuous M-6-P treatment at 100 μ M. The media was

refreshed every three to four days and the coverslips harvested every seven days. The coverslips were fixed in methanol and then frozen; and then immunostained for α -smooth muscle actin and the proportional myofibroblasts counted under the microscope, under an X 40 objective. Propidium iodide was used as a nuclear counterstain.

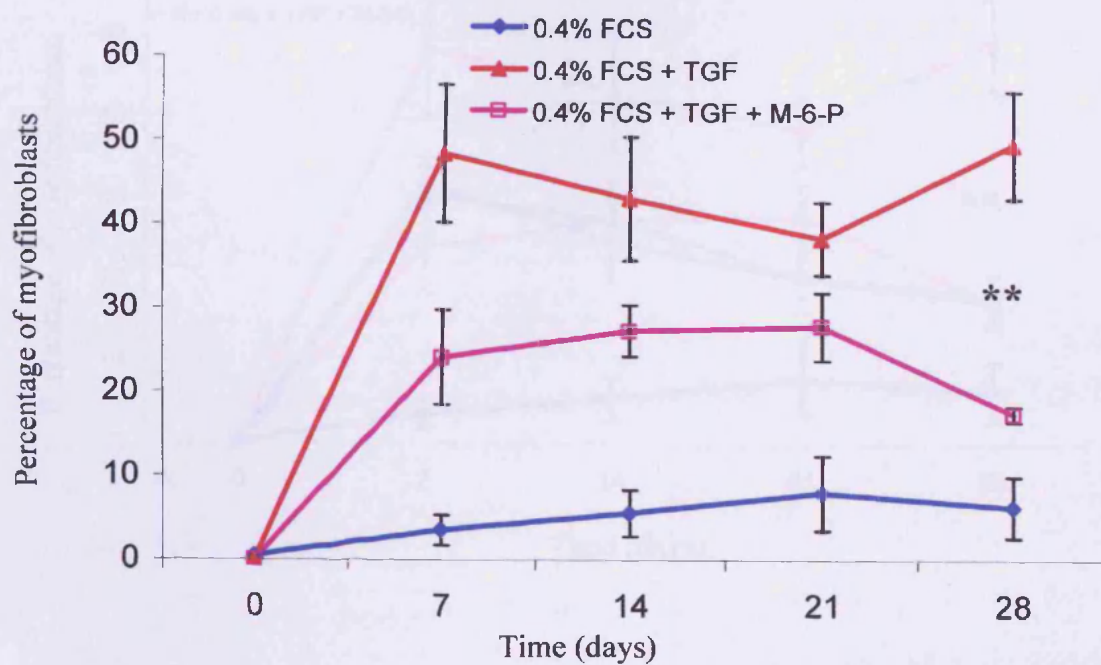
The percentages of myofibroblasts in the cell cultures were calculated for each time point and plotted (see figs. 5.2.1 a – d). The untreated fibroblasts show less than 10% myofibroblast differentiation throughout the time course (figs. 5.2.1 a-d). The fibroblasts exposed to three days' treatment with TGF- β 1 demonstrated greater myofibroblast differentiation reaching a plateau of approximately 50% after seven days and did not significantly drop over 28 days (figs. 5.2.1 a-d). In contrast the insulin-treated fibroblasts displayed reduced differentiation overall, even shortly after TGF- β 1 treatment being only 30% at seven days, which then declines further over the rest of the time course to less than 20% (figs. 5.2.1 a and d) at day 28 and is significantly lower than that seen with TGF- β 1 treatment alone ($p < 0.05$).

The fibroblasts exposed to three days of TGF- β 1 plus continuous M-6-P in an attempt to block activation of autocrine TGF- β 1, behave in a remarkably similar fashion to the insulin-treated cells with a peak between days 7 and 21 at approximately 30% followed by a drop to less than 20% by day 28 which was significantly lower than that seen in TGF- β 1 alone treated cells ($p < 0.05$). This M-6-P-treated cell curve was not significantly different from the insulin-treated curve (fig. 5.2.1 a) at any time point.

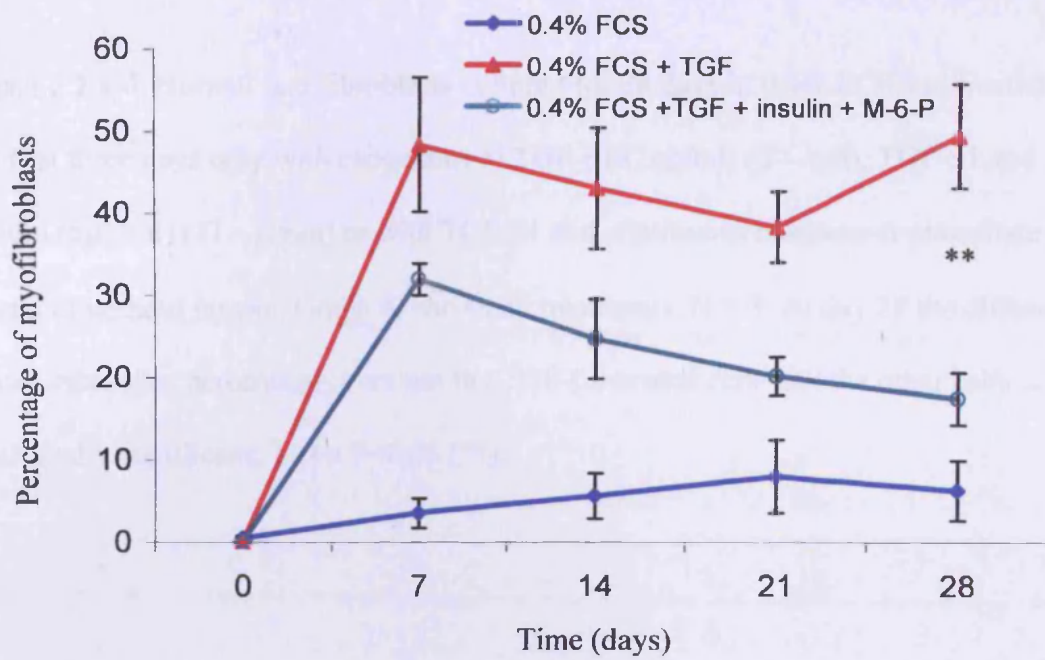
The fibroblasts treated with TGF- β 1 and insulin for three days and continuous M-6-P behaved in an indistinguishable fashion to that of TGF- β 1 and insulin-treated cells and the TGF- β 1 and M-6-P treated cells showing a peak of differentiation at seven days, at a reduced level of about 30% followed by a gradual decline in differentiation to less than 20% by day 28 which was significantly lower ($p<0.05$) than the TGF- β 1 only treated cells at this time point (figs. 5.2.1 c & d).



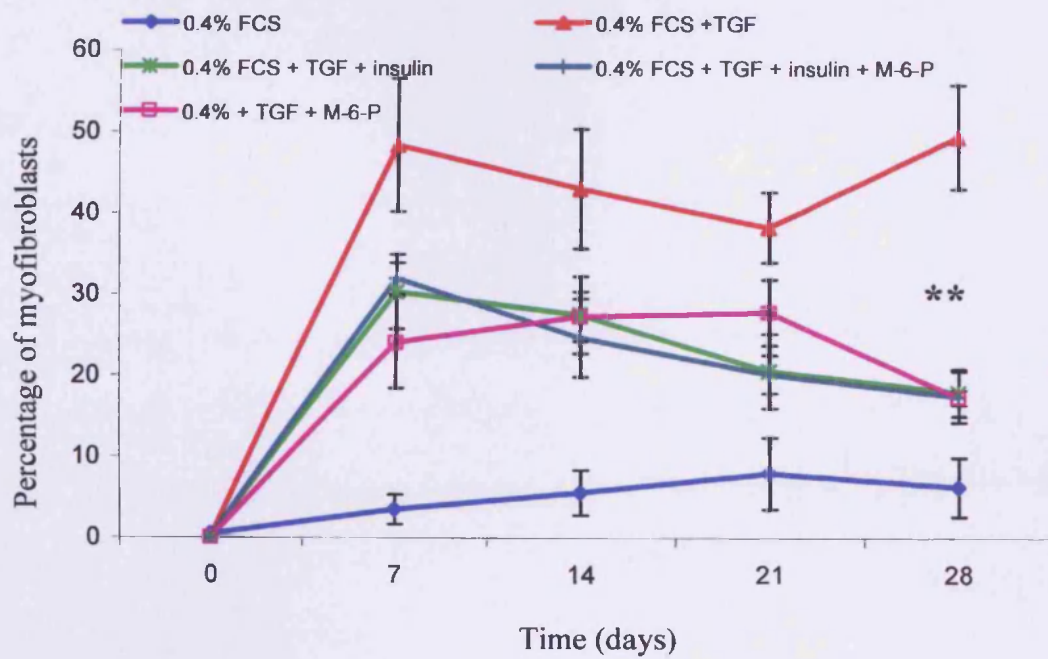
a)



b)



c)



d)

Figs.5.2.1 a-d. Normal scar fibroblasts cultured for 28 days in 0.4% FCS and treated for the first three days only with exogenous a) TGF- β 1 (2ng/ml) (T – red), TGF- β 1 and insulin (5 μ g/ml) (TI – green) or with TGF- β 1 and continuous mannose-6-phosphate with b) and c) without insulin. Graph d) shows all treatments. N = 3. At day 28 the difference in myofibroblast percentages between the TGF- β 1 treated cells and the other cells is statistically significant; T-test $P < 0.05$ (**).

5.2.3 Discussion

These results (fig. 5.2.1 a) confirm that exogenous TGF- β 1 has a pro-myofibroblast differentiating affect beyond its period of treatment and that the initial induction of myofibroblast differentiation by TGF- β 1 is not significantly affected by insulin. However, after the TGF- β 1 and insulin treatments are withdrawn the insulin-treated cells demonstrate a significant drop in the number of myofibroblasts that continues up to day 28 (fig. 5.2.1 a).

M-6-P does not appear to inhibit the initial myofibroblast differentiation affected by exogenous TGF- β 1, which is in an activated form, but does inhibit myofibroblast differentiation after the TGF- β 1 is withdrawn and furthermore, this inhibition appears identical to that seen in the insulin-treated cells (figs. 5.2.1 a and c). This behaviour in the M-6-P-treated cells confirms the hypothesis that the prolonged presence of myofibroblasts is due to autocrine TGF- β because M-6-P inhibits activation of latent TGF- β 1 whereas the exogenous TGF- β 1 is already in an activated form and thus M-6-P does not inhibit the initial myofibroblast differentiation in response to exogenous TGF- β 1 (fig. 5.2.1 b). Once the exogenous TGF- β 1 is withdrawn the maintenance of high levels of myofibroblasts must therefore be due to the effects of autocrine TGF- β 1, and the effects of insulin are therefore likely to be via affecting autocrine TGF- β .

It must be noted that these experiments have taken place in 0.4% FCS containing medium. They were performed in this medium for two reasons: firstly to ensure minimal disruption by differentiation inhibitory factors present in serum and secondly

because cultures show very little proliferation in this medium thus removing the factor of increases in cell numbers from the equation. The connotations of this are that the long-term maintenance of high proportions of myofibroblasts in TGF- β 1 pulse-treated cells could be due to: 1) autocrine TGF- β 1 maintaining a steady population of myofibroblast phenotype cells – but if removed these cells disappear via apoptosis (unlikely as insulin inhibits apoptosis) or via de-differentiation; or 2) that there is a continual cycling of cells turning into myofibroblasts that is driven by autocrine TGF- β

The insulin, TGF- β 1 and M-6-P treated cells (fig. 5.2.1 c) show that insulin and M-6-P do not have additive effects, which suggests that they do not inhibit myofibroblast differentiation by different pathways; that is insulin exerts its' effect via a similar mechanism as M-6-P, which we know inhibits the activation of autocrine TGF- β 1. However the exact mechanism at the molecular level is not clarified by this experiment, as insulin is known not to be a ligand of the IGF-II receptor (Wardzala et al., 1984) and therefore does not work in the same way as M-6-P, that is, by competitive inhibition of the M-6-P binding site of which latent TGF- β 1 is a ligand. This issue will be addressed later in this chapter (see section 5.6).

5.3 Does insulin affect the levels of latent TGF- β 1?

5.3.1 Introduction

To further explore the effect of insulin on the activation of TGF- β 1, the effects of the interaction between insulin and exogenous latent TGF- β 1 on myofibroblast differentiation can be explored using a commercially available antibody specific for TGF- β 1 and latent TGF- β 1. In simple terms if insulin is inhibiting activation of autocrine TGF- β then we would expect to detect higher amounts of latent TGF- β 1 in insulin-treated fibroblast cultures. Testing this hypothesis involves investigation of insulin's effect on latent TGF- β 1 in terms of immunostaining and determination of the levels of the protein itself by Western blotting. The exact size of the latent TGF- β 1 depends on which latent proteins it is associated with and whether it is a dimer. All of the different forms would normally be expected to be expressed and secreted by the cells provided they have functioning, activating receptors. More instructive than the absolute levels of each of the TGF- β forms is the ratio of their levels and these will be sought in this section to see whether insulin can affect them and thus affect inhibition of myofibroblast differentiation by tilting the ratio in favour of increased inactivated TGF- β .

TGF- β 1 LAP is a ubiquitously expressed molecule (Wakefield et al, 1987) so we might expect all of cells to show some degree of staining.

5.3.2 Results

Normal scar fibroblasts were cultured on coverslips for two weeks in DFCS and subsequently fixed in formaldehyde as per data sheet instructions before immunostaining with anti-LAP TGF- β 1 antibody (R & D Systems) and examination under a X 40 objective on a fluorescent microscope.

Most cells did not detectably stain for LAP but those that did showed a diffuse green fluorescence throughout the cytoplasm (examples shown below) and such cells were counted as positive and the proportion of positive cells calculated. These results are shown below. There is a trend towards increased LAP presence (40% over that of untreated) in the insulin-treated cells (see figs. 5.3.1 and 2) although this difference is non-significant.

For the determination of protein expression of latent TGF- β 1 normal scar fibroblasts cultured in DTTM and DFCS with and without insulin (5 μ g/ml) for 14 days in T75 flasks. The cells at day 0 were seeded at 7.5×10^5 and by day 14 were near confluence. Media was refreshed twice weekly. The protein lysate was prepared from the flasks as previously described (see section 2.5.1). Western blotting analysis was undertaken using an anti-LAP antibody (R & D Systems) with anti-GAPDH for comparison (see figs. 5.3.5-6). In addition, Western blot analysis was additionally performed using anti-LTBP (TGF- β 1 large latent complex) (R & D Systems) for confirmation of large latent complex analysis (fig. 5.3.7).



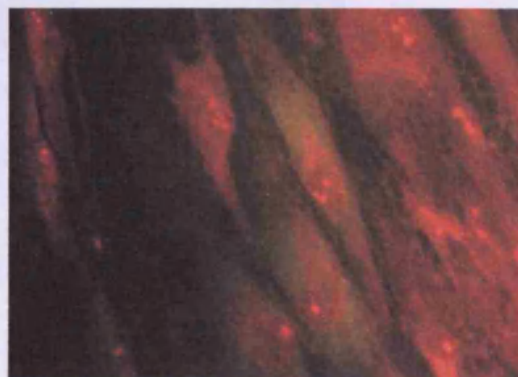
a) DTTM



b) DTTM plus insulin



c) DFCS



d) DFCS plus insulin

Fig. 5.3.1 a-d Normal scar fibroblasts cultured in a) & b) DTTM and c) & d) DFCS with and without insulin (5 μ g/ml). Immunostained on day 14 with anti-latency associated protein fluorescence conjugated antibodies with a propidium iodide counterstain.

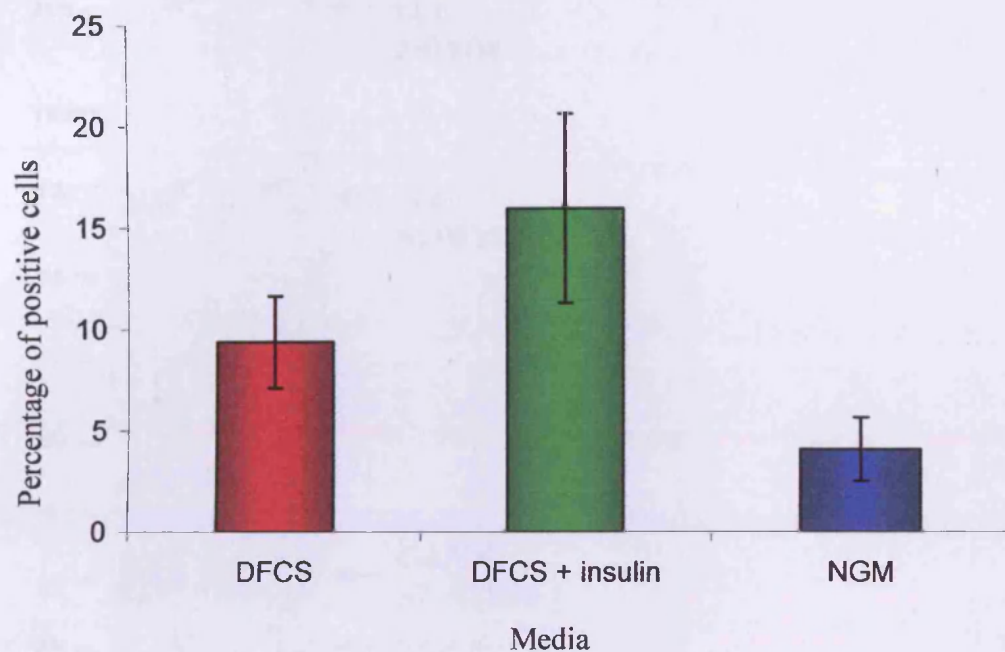


Fig. 5.3.2 Normal scar fibroblasts cultured in DFCS with and without insulin (5 μ g/ml). Immunostained on day 14 with anti-latency associated protein fluorescence conjugated antibodies with a propidium iodide counterstain. N = 3. T-test NGM vs. treated P=0.168, NGM vs. untreated P=0.138, treated vs. untreated P=0.252.

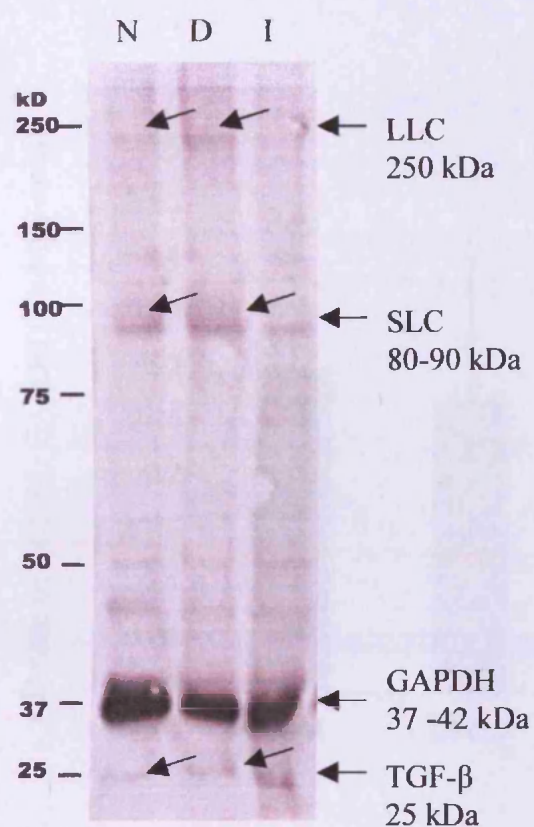


Fig. 5.3.3 A typical Western blot demonstrating the active TGF- β dimer band at 25kDa, the SLC at 80-90kDa and the LLC at just below 250kDa. (N – NGM, D – DFCS, I – DFCS + insulin).

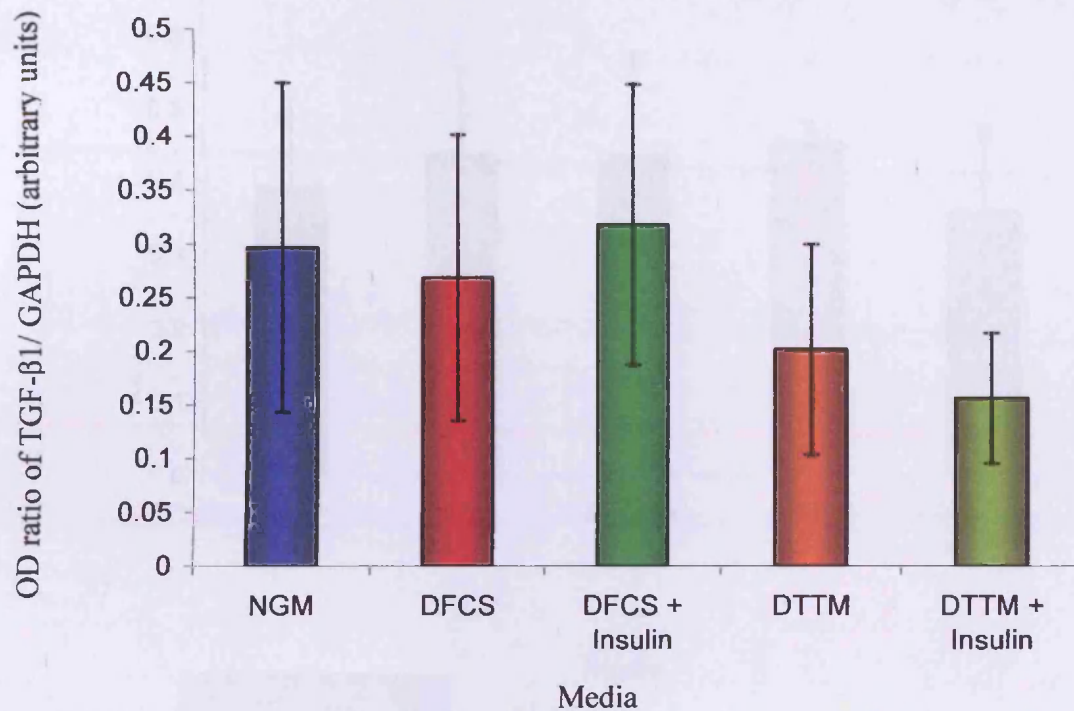


Figure 5.3.4 Densitometry analysis of western blots for TGF-β1 (25kDa) taken from normal scar fibroblasts cultured in DTTM and DFCS with and without insulin (5μg/ml) for 14 days. N=4. Kruskal-Wallis One Way Analysis of Variance on Ranks P=0.908. Error bars represent standard errors of the mean

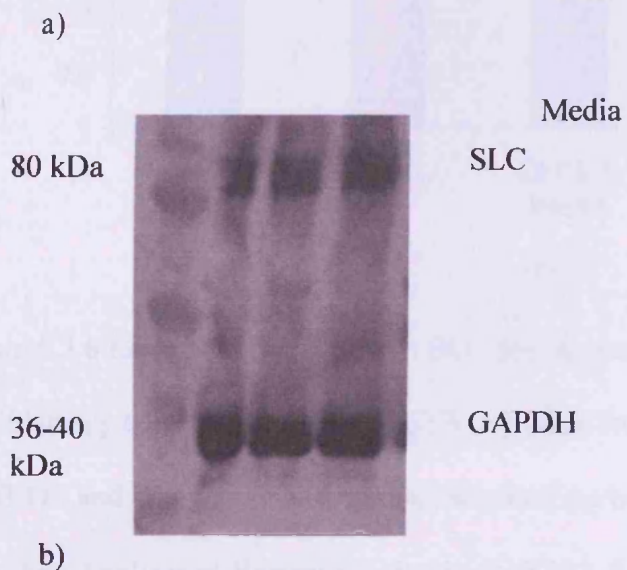
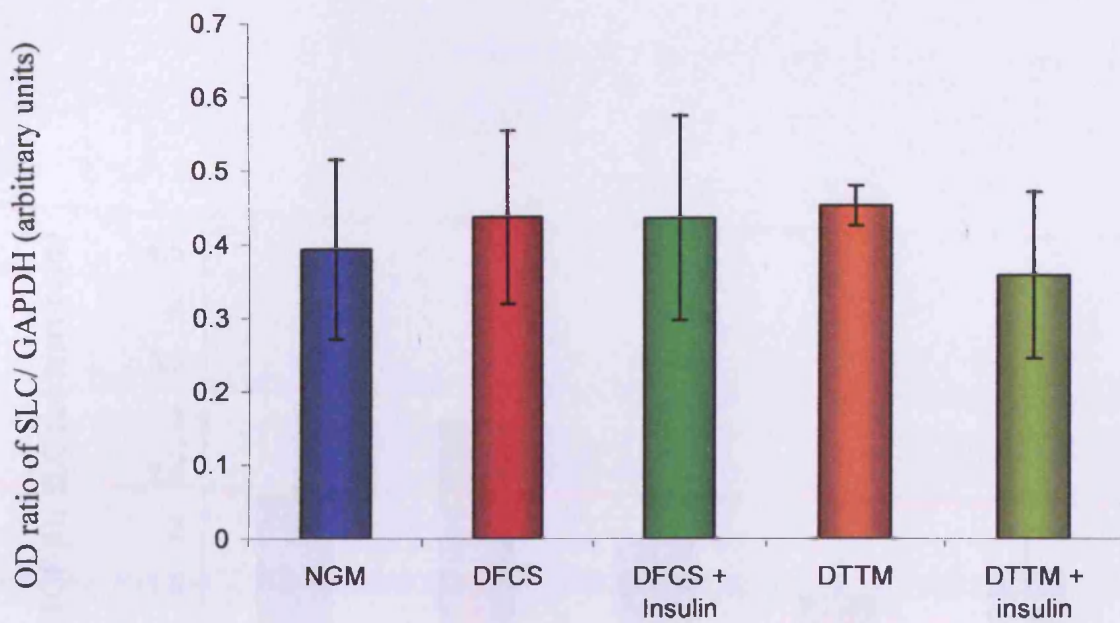


Figure 5.3.5 a) Densitometry analysis of western blots for the small latent complex (80kDa) taken from normal scar fibroblasts cultured in DTTM and DFCS with and without insulin (5 μ g/ml) for 14 days. N=4. Kruskal-Wallis One Way Analysis of Variance on Ranks P=0.978. Error bars represent SEM. b) Representative Western blot showing bands at 80 kDa for the small latent complex and 36-40 kDa for GAPDH.

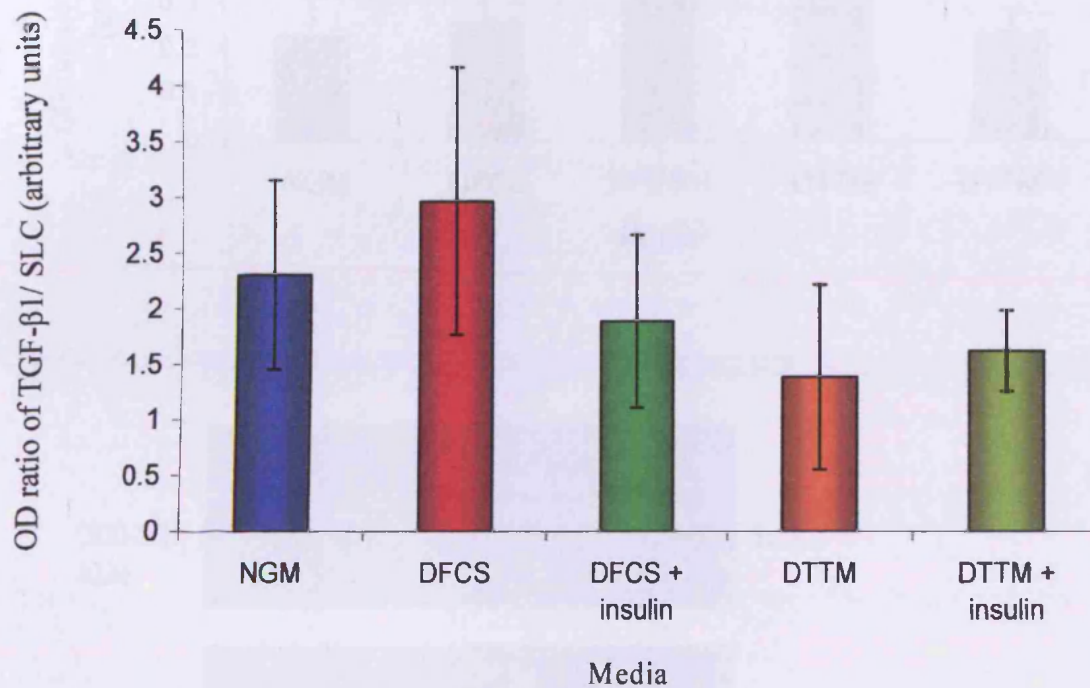
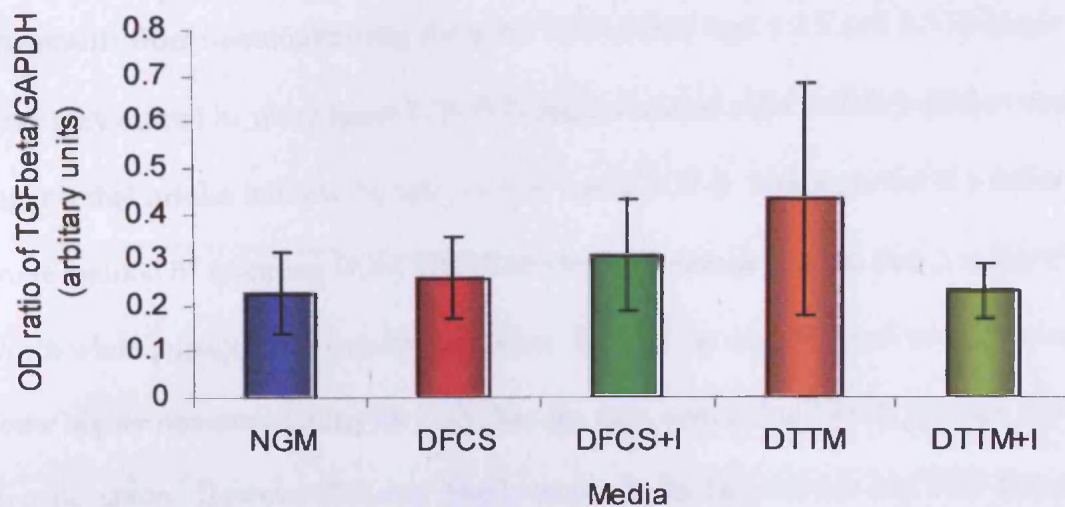
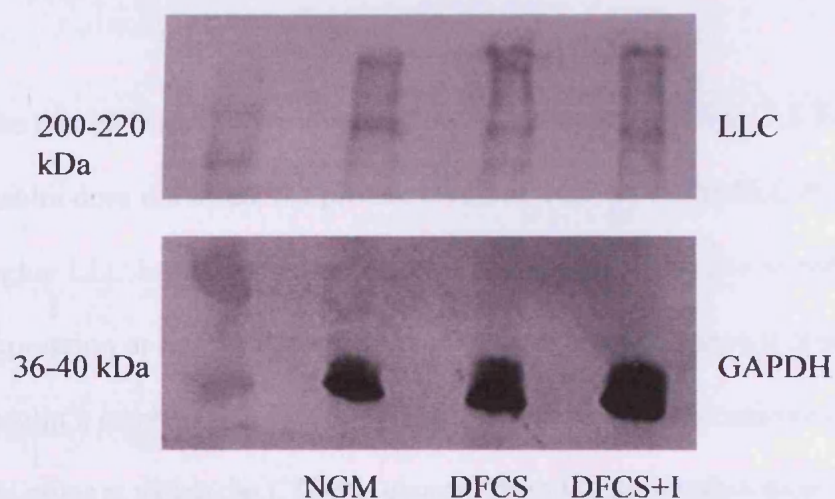


Figure 5.3.6 Ratio of active TGF-β1: SLC densitometry analysis of western blots for the small latency complex and TGF-β1 (25kDa) taken from normal scar fibroblasts cultured in DTTM and DFCS with and without insulin (5μg/ml) for 14 days. N=4. Kruskal-Wallis One Way Analysis of Variance on Ranks P=0.752. Error bars represent SEM.



a)



b)

Figure 5.3.7 a) Densitometry analysis of western blots for the large latency complex (220 kDa) taken from normal scar fibroblasts cultured in DFCS and DTTM with and without insulin (5 μ g/ml) for 14 days. Error bars represent SEM. Kruskal-Wallis One Way Analysis of Variance on Ranks $P=0.804$. b) Representative Western blots showing bands at 200-220 kDa for the large latent complex and 36-40 kDa for GAPDH. $N = 5$ cell strains.

5.3.3 Discussion

The results from immunostaining for latent TGF- β (see figs. 5.3.1 and 5.3.2) imply that there may indeed be more latent TGF- β in insulin-treated cells, which would in turn suggest that insulin inhibits the activation of latent TGF- β . Although this is a rather crude method of assessing latent TGF- β levels, these results suggest that it is therefore worth while pursuing this hypothesis further. Both of the experimental media appear to show higher immunostaining for LAP than the cells cultured in NGM, perhaps contrary to expectation. However this may simply be due to the fact there is less TGF- β overall, active and inactive, present in the NGM-treated cells although this was not the case for the levels of TGF- β (determined by ELISA) secreted into the medium (see fig. 4.4.1).

The results from the Western blotting experiments (see figs. 5.3.5-6) indicate that insulin does not affect the protein levels of TGF- β 1 or the SLC but may contribute to higher LLC levels in insulin treated cells. This could be due to either greater TGF- β expression or less LLC activation. If this is a real trend then it would suggest that insulin's mechanism of action is via the inhibition of activation of autocrine TGF- β 1 at the point at which the LTBP is cleaved from the SLC rather than the point at which the active TGF- β 1 is released from the SLC following binding to the IGF-II receptor. However, the exact nature of LLC activation is unknown. It could occur via plasmins, acidic microenvironments or phosphatases but not necessarily by the IGF-II receptor (Miyazono et al. 1991). The SLC on the other hand does bind and is activated by the IGF-II receptor (Miyazono et al. 1991).

This uncertainty could perhaps be clarified by repeating the experiment described in section 5.2 but this time measuring the protein levels of active TGF- β 1, SLC and LLC in both the insulin and M-6-P treated fibroblasts. It is known that M-6-P inhibits the activation of latent TGF- β 1 action via blocking binding of the SLC to the IGF-II receptor so it would be interesting to see how much of an effect this actually has on the protein levels of the SLC and LLC and compare the findings with those for insulin. It may be that the effects of M-6-P on the relative levels of each form of TGF- β are actually only very small which would cast a better light on the current results.

Comparison of the TGF- β 1 and SLC bands (fig. 5.3.6) indicates a 36% drop in the insulin treated cells. The relative levels of these molecules are more instructive than their absolute amounts and this finding, though not significant, suggests that it is still possible that insulin is decreasing activation of the SLC.

Overall the results do not suggest a statistically significant effect of insulin on the expression of activated or inactivated TGF- β 1 although there is a suggestion that LLC levels are higher in insulin treated fibroblasts.

5.4 Does insulin affect autocrine TGF- β activity?

5.4.1 Introduction

The studies presented so far have assessed TGF- β in terms of either mRNA levels, immunostaining, protein expression or changes in myofibroblast numbers but these are all really surrogate measures of its most important attribute, namely its activity.

Conclusions about the inhibition of autocrine TGF- β action have necessarily been inferred rather than measured. This is in large part due to the difficulty of performing a TGF- β activity assay. However, performing such an assay will allow either a refutation of the autocrine TGF- β hypothesis or demonstrate its potential as insulin's mechanism of action and as such is a crucial investigation. Attempts were made to measure levels of active TGF- β using the ELISA technique described in section 4.4 to measure total TGF- β . In this instance the ELISA was attempted in the absence of the acidification used to activate latent TGF- β , however the assay proved too insensitive. A more sensitive bioassay was therefore employed (see section 2.6 for method).

5.4.2 Results

Normal scar fibroblasts were cultured in DFCS with and without insulin (5 μ g/ml) for 14 days at which point the media were collected and subjected to the mink lung epithelial cell (MLEC) Luciferase assay (see section 2.6) in order to determine total TGF- β and active TGF- β levels from which the inactive TGF- β levels can be calculated. The results indicate that although insulin does not inhibit secretion of autocrine TGF- β 1 (fig. 5.4.1), it is significantly ($P < 0.03$) associated with reduced levels of active TGF- β (fig. 5.4.2).

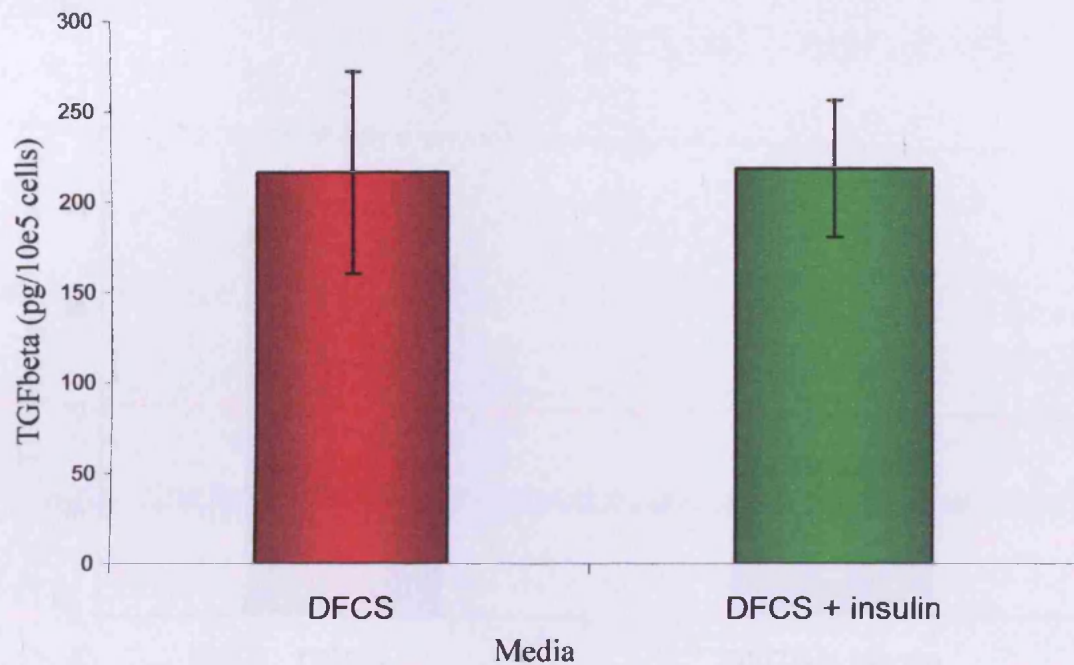


Figure 5.4.1 Quantification of total TGF- β (MLEC Luciferase assay – see section 2.6) secreted by normal scar fibroblasts cultured in DFCS with and without insulin (5 μ g/ml) for 14 days. N = 3 normal scar cell strains. No significant difference between untreated and treated groups, P = 0.96. Error bars represent SEM.

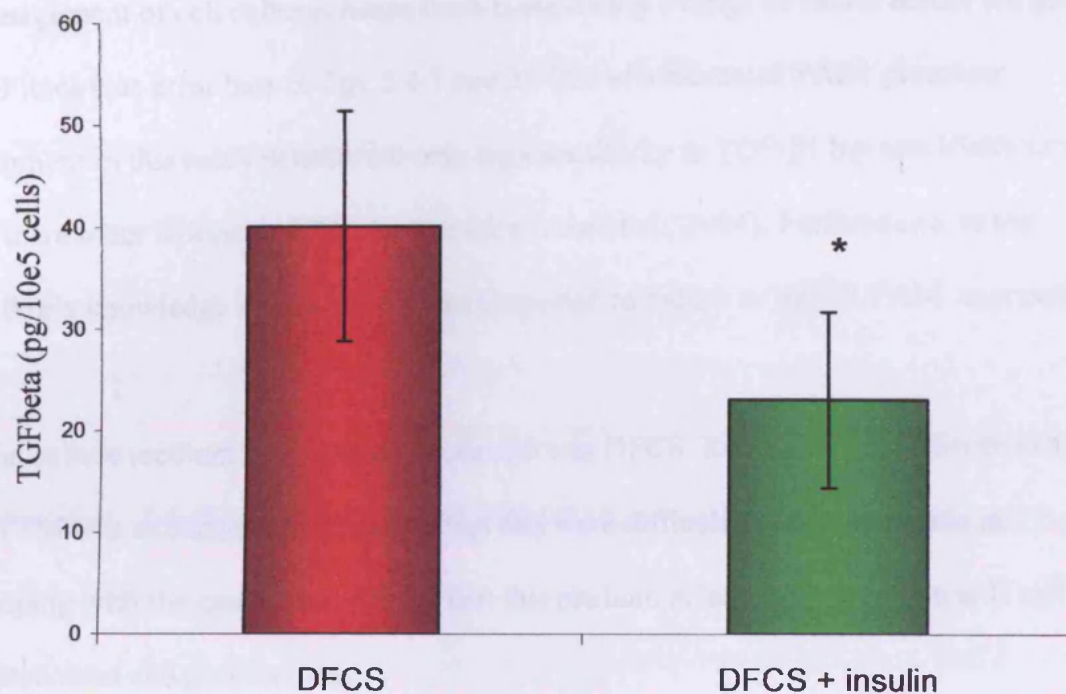


Fig. 5.4.2 Quantification of active TGF β (MLEC Luciferase assay – see section 2.6) secreted by normal scar fibroblasts cultured in DFCS with and without insulin (5 μ g/ml) for 14 days. N = 3 normal scar cell strains. Error bars represent SEM. P < 0.03 (*).

5.4.3 Discussion

This experimental technique is complex requiring multiple steps and careful management of cell cultures hence there is inevitably a range of values across the three cell lines (see error bars in figs. 5.4.1 and 2). Use of a truncated PAI-1 promoter fragment in this assay permits not only high sensitivity to TGF- β 1 but specificity too, as there are other inducers of PAI-1 expression (Abe et al., 1994). Furthermore, to the author's knowledge insulin has not been reported to induce or inhibit PAI-1 expression.

The culture medium used in this experiment was DFCS. Repetition of the experiment in DTTM was attempted but gave readings that were difficult to detect which is in keeping with the general impression that this medium is less favourable towards cell metabolism and proliferation.

Despite the variability between cell lines, the finding that insulin does not inhibit the production or secretion of autocrine TGF- β 1 (fig. 5.4.1) but is associated with decreased levels of active TGF- β (by approximately half) in the media of insulin treated fibroblast cultures (fig. 5.4.2) is highly significant ($p < 0.03$). The implication is that insulin inhibits the activation of autocrine TGF- β 1. These results are consistent with the rest of the findings in this thesis, namely: insulin does not inhibit the mRNA levels or protein production of TGF- β 1, insulin is associated with trends towards a reduced active TGF- β 1/SLC ratio and finally that insulin appears to inhibit the action of autocrine TGF- β 1 in a similar way to mannose-6-phosphate. This sequence clearly

points to both the $\alpha V\beta 5$ integrin and the M-6-P/IGF-II receptor being the next subjects of investigation (see section 5.5 and 6).

These results are also consistent with the finding that insulin does not inhibit myofibroblast differentiation when exogenous TGF- $\beta 1$ is added to the culture medium even after prolonged insulin treatment (14 days – see fig. 3.4.2). This is because exogenous TGF- $\beta 1$ is already activated and therefore not susceptible to insulin's mechanism of reducing TGF- $\beta 1$ activation. It also explains why insulin did not reduce myofibroblast numbers to zero in the wounds in the murine model (Mackie, 2004) - the initial induction of myofibroblast differentiation by paracrine TGF- $\beta 1$ is completely unaffected by insulin. Further, it is consistent with the finding that in the absence of TGF- $\beta 1$ treatment, insulin treated cultures still generate myofibroblasts, albeit fewer, because insulin does not eliminate TGF- $\beta 1$ activity altogether but simply reduces it. In conclusion, this is an important result that gives rationale to the in vitro work presented so far.

5.5 Does insulin affect the expression of $\alpha V\beta 5$ integrin?

5.5.1 Introduction

Integrins regulate cell adhesion to the extracellular matrix and signalling between the two and are intimately involved in wound healing and scar formation (Beanes et al. 2003). There are currently 24 different integrin subunits with many of them having overlapping and multiple or unclear roles. For the purposes of practicality therefore, only the integrin of particular importance in latent TGF- β activation in fibroblasts was concentrated upon, that is $\alpha V\beta 5$. All αV subunits of integrin are known to bind to the small latent TGF- β complex and modulate its location and possibly activation (Munger et al. 1999; Mu et al. 2002), however $\alpha V\beta 5$ specifically has been shown to specifically recruit and activate autocrine TGF- $\beta 1$ on the surface of systemic sclerosis fibroblasts (Asano et al. 2005) whereas other αV -containing integrin dimers that have been shown to activate latent TGF- β are expressed on other cell types. Anti- $\alpha V\beta 5$ antibodies reduce TGF- $\beta 1$ -induced $\alpha 2(I)$ collagen gene promoter activity, inhibit activation of the SLC and reverse the morphological myofibroblastic features of fibroblasts (Asano et al. 2005). $\beta 5$ antisense oligonucleotides reduce phosphorylation levels of SMAD 3 and its DNA binding ability (Asano et al. 2005). $\alpha V\beta 5$ integrin may achieve activation of the SLC in similar way to the epithelially expressed $\alpha V\beta 6$ as it has a cytoplasmic domain that is strikingly homologous with the same domain in the $\beta 6$ subunit (Asano et al. 2005).

Thus if insulin were shown to inhibit the expression of this integrin, then this would indicate a possible mechanism of action via reduced activation of TGF- β 1. This section seeks to explore this possibility.

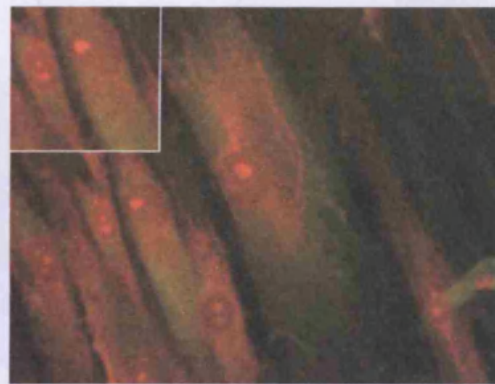
5.5.2 Results

Normal scar fibroblasts were cultured on coverslips in NGM, DFCS with and without insulin (5 μ g/ml), and DTTM with and without insulin (5 μ g/ml). At 14 days they were fixed in 10% formaldehyde as per data sheet instructions and immunostained using anti- α V β 5 integrin antibodies with propidium iodide as the nuclear counterstain. The coverslips were examined under a light microscope (x40 magnification) and those fibroblasts that demonstrated obvious anti-integrin immunostaining were counted as 'positive' and expressed as a percentage of the total number of fibroblasts.

A proportion of the fibroblasts in all of the media stained positively for α V β 5 integrin at levels of between 15 and 20% (see figures 5.5.1.a and b and 5.5.2). There was no statistically significant difference in the percentage of α V β 5 positive cells between the untreated and insulin-treated cells ($P=0.365$). Nevertheless, the numbers of positive fibroblasts in DTTM plus insulin (but not DTTM only) were significantly higher ($p>0.05$) than that seen in NGM although the significance of this finding is unclear at this time.



a) DFCS



b) DFCS plus insulin

Fig. 5.5.1 a and b. Typical $\alpha V\beta 5$ integrin immunostaining (green) of normal scar fibroblasts counterstained with propidium iodide, a) DFCS b) DFCS plus insulin (5 μ g/ml).

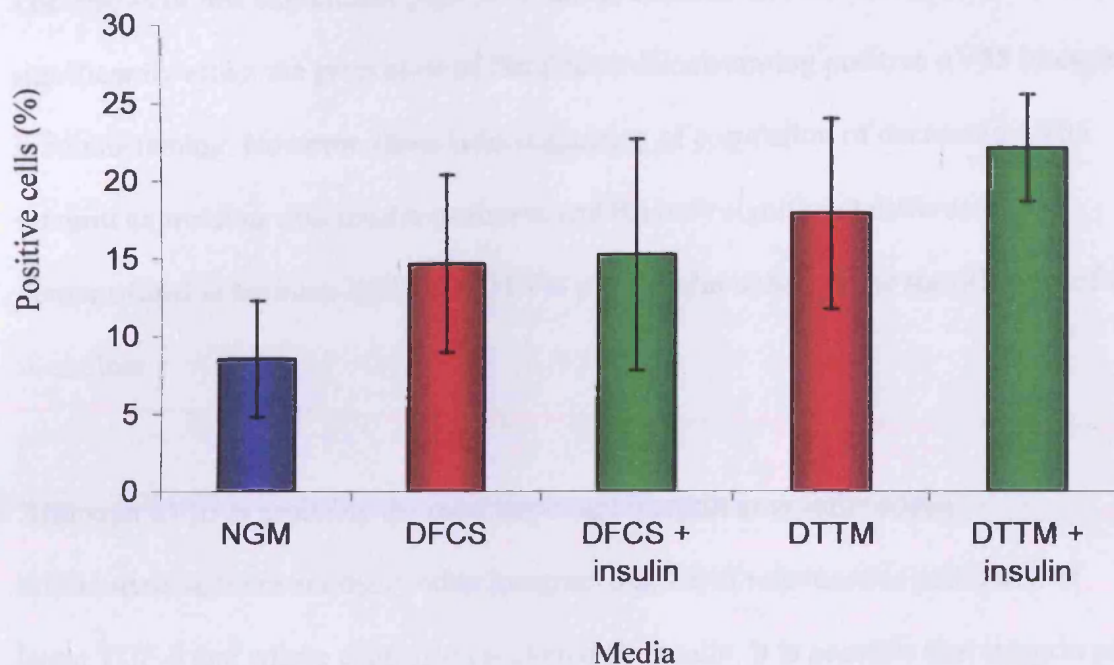


Fig. 5.5.2. The percentage of normal scar fibroblasts staining positively with anti- α V β 5 integrin antibodies after culture for 14 days in DFCS and DTTM with and without insulin. N = 4 cell strains. Error bars represent SEM. Kruskal-Wallis One Way Analysis of Variance on Ranks P = 0.365. T-test – DTTI compared with NGM P=0.05, whereas DTT compared with NGM P=0.23); other T-test comparisons P> 0.4.

5.5.3 Discussion

The results of this experiment (figs 5.5.1 and 2) indicate that insulin does not significantly affect the proportion of fibroblasts demonstrating positive $\alpha V\beta 5$ integrin immunostaining. However, there is no suggestion of correlation of decreased $\alpha V\beta 5$ integrin expression with insulin treatment and the only significant difference demonstrated is between NGM and DTTM plus insulin although the significance of this is unclear.

Although $\alpha V\beta 5$ is probably the most important integrin in myofibroblast differentiation, there are many other integrins that are of relevance to activation of latent TGF- β and whose expression is altered by insulin. It is possible that integrin pairs not normally expressed by fibroblasts might be expressed on wound healing or scar-forming fibroblasts. For instance, in addition to $\alpha V\beta 5$, $\alpha V\beta 1$ integrin also directly binds to the latency-associated peptide of TGF- $\beta 1$ (Munger et al. 1999). Another example is $\alpha V\beta 6$ integrin, albeit normally restricted to epithelial cells, which also binds the TGF- $\beta 1$ LAP again via the RGD motif (demonstrated by its inability to bind LAP- $\beta 1$ when it is in a mutant RGE form), but in contrast with $\alpha V\beta 1$ it does activate latent TGF- $\beta 1$ and this activation can be blocked with $\alpha V\beta 6$ antibodies (Shepherd 2005). Uniquely however $\alpha V\beta 6$ integrin activates TGF $\beta 1$ without releasing the free TGF- β from the latent complex (Annes et al. 2004). In fact $\alpha V\beta 6$ activates the LAP only in a paracrine fashion and requires direct cell contact. $\alpha V\beta 6$ also binds and activates TGF- $\beta 3$ (Shepherd 2005). Integrin $\alpha V\beta 8$ has also been shown to bind to LAP and activate it,

despite having a completely different cytoplasmic domain to $\alpha V\beta 6$ (Mu et al. 2002). $\alpha v\beta 8$ also appears to activate TGF- β in a different way to $\alpha V\beta 6$ by presenting the latent complexes to cell surface metalloproteases, which in turn releases free diffusible TGF- β into the extracellular fluid (Mu et al. 2002).

As integrin activity is so closely entwined with activation of TGF- β , inhibition of integrins by insulin could potentially be an effective mechanism of action for insulin. However, with their overlapping roles it might also mean that insulin would have to have an inhibitory effect across the whole class to be truly efficacious. With several integrins involved in TGF- β activation it could be revealing to examine insulin's interactions with some of the other integrins, particularly $\alpha V\beta 1$, $\alpha V\beta 6$ and $\alpha V\beta 8$.

5.6 Does insulin affect the expression of the IGF-II receptor?

5.6.1 Introduction

The results so far suggest that insulin may inhibit myofibroblast differentiation by inhibiting the activation of the latent forms of autocrine TGF- β 1 via a similar mechanism to M-6-P, that is, an interaction with the IGF-II receptor. This section therefore examines whether an effect on the IGF-II receptor in response to insulin treatment can be demonstrated. The next question to answer therefore is ‘what is the nature of the interaction between insulin and the IGF-II receptor?’

As previously highlighted, it is known that any possible interaction between insulin and the IGF-II receptor does not involve a direct ligand-receptor binding (Kasuga et al. 1981; Massague et al. 1981). Therefore insulin may utilise other less direct means of inhibiting the receptor’s actions such as reducing its cell surface expression, altering its cell surface-intracellular distribution, interacting with the intracellular signalling pathway or perhaps altering the relative affinities of the IGF-II receptor for its different ligands, or altering the expression of alternative ligands that enhance or undermine the receptor’s TGF- β 1 activating activity. Due to limitations of time the examination of all of these possibilities is beyond the limit of this thesis. Instead the next series of experiments concentrates on examining insulin’s effects on the IGF-II receptor in terms of its cell surface expression/distribution (immunostaining and microscopy), mRNA expression (RT-PCR) and finally the receptor’s protein expression (Western blotting).

Immunostaining of the IGF-II receptor has been performed by groups studying fibroblasts (Brown 1990), osteoclasts (Baron et al. 1990), hepatocytes and ovaries (Brown et al. 1987). Using immunochemistry Brown (1990) found that the cation independent M-6-P receptor in normal human skin fibroblasts was concentrated in the trans Golgi cisternae and at the cell surface with the receptors recycling between the two locations. The Golgi network seems to be important in not just the storage of the receptor but also its phosphorylation before transport to the cell surface (Meresse et al. 1993) where these authors noted a distinct punctuate pattern of immunostaining of the receptor. The purpose of analysing the receptor by immunochemistry and microscopy in this research is to reveal whether there any differences between insulin-treated and untreated fibroblasts in terms of the cell surface distribution/pattern of IGF-II receptors and whether there are any quantifiable differences such as proportions of positively staining cells or noticeable differences in intensity of staining.

The human IGF-II receptor mRNA expression has been previously studied in neurons in relation to Alzheimer's disease (Rivera et al, 2007) and IGF-I have been studied in human keratinocytes in the context of hypertrophic burn scars (Ghahary et al. 1998). It is unknown whether insulin affects transcription of IGF-II receptor gene. If insulin were found to reduce the expression of IGF-II receptor mRNA, then this would provide a plausible mechanism of action for insulin.

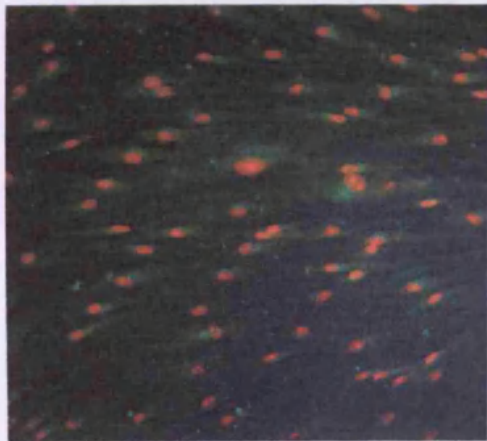
Finally IGF-II receptor protein expression has been previously studied in human fibroblasts (Bräulke et al., 1987) and insulin is known to cause redistribution and

increased cellular degradation of the receptor in rat adipocytes (Oka et al., 1985) although to the author's knowledge insulin's affect on the protein expression of IGF-II receptor in human fibroblasts has not been studied. This will be examined in this section by Western blotting.

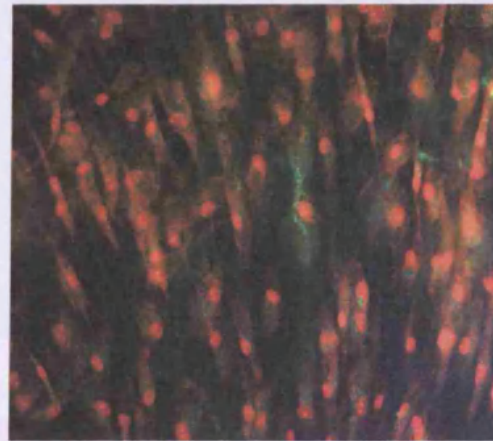
5.6.2 Results

IGF-II receptor immunohistochemistry

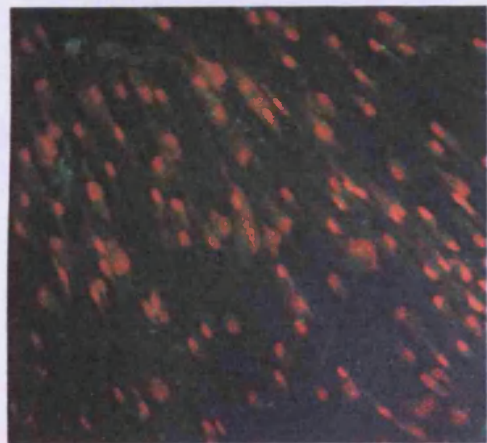
Normal scar fibroblasts were cultured on coverslips for 14 days in dialysed FCS with and without insulin and harvested at the end of this time course, fixed with cold methanol and immunostained with anti-human IGF-II receptor antibodies (R & D Systems) with a propidium iodide counterstain. The pattern of staining was noted. Most cells were found not to show any distinct staining whereas a minority showed overt staining (see fig. 5.6.1) and these fibroblasts were identified as 'positive' and calculated as a percentage of the total show in figure 5.6.2. The IGF-II receptor immunostaining revealed a characteristic staining pattern of perinuclear speckling in the positively-staining fibroblasts (see fig. 5.6.1). Furthermore, the proportion of cells demonstrating this staining was consistently lower in the insulin treated cells although this did not quite reach significance ($P=0.144$) (see fig. 5.6.2). Interestingly, occasional cells were also seen with bright immunostaining in discrete intracytoplasmic organelles (see arrow and inset image in fig. 5.6.1). Nevertheless the appearance of these cells was relatively rare (approximately 1%) and did not appear to differ between treatments.



a) DFCS



b) DCFS and insulin



c) NGM

Figs. 5.6.1 a-c. Normal scar fibroblasts cultured in DFCS with and without insulin (5 μ g/ml) and in NGM for comparison. Immunostained on day 14 with anti-IGFII receptor fluorescence conjugated antibodies (green) with a propidium iodide counterstain (orange). A to c above are representative photographs taken at x 40 magnification.

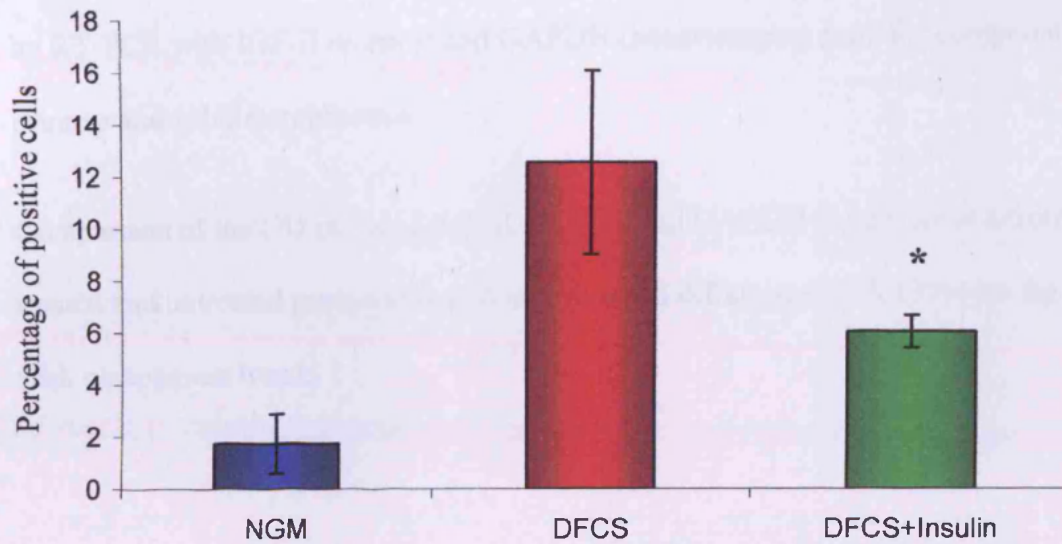


Figure 5.6.2 Normal scar fibroblasts cultured in DFCS with and without insulin (5 μ g/ml) and in NGM for comparison. N = 3. Error bars represent SEM. T-test comparison of DFCS with DFCS and insulin, P = 0.144; DFCS and insulin with NGM, P=0.035 (*); DFCS and NGM, P=0.102.

IGF-II receptor mRNA levels

Normal scar fibroblasts were cultured in DFCS with and without insulin (5 μ g/ml) in 6 well plates for 17 days at which time they were subjected to RNA extraction followed by RT-PCR with IGF-II receptor and GAPDH (housekeeping gene for comparison) primers and gel electrophoresis.

Comparison of the OD ratios of IGF-II receptor and GAPDH bands derived from the treated and untreated groups revealed no statistical difference ($P=0.139$) (see fig. 5.6.3) with no apparent trend.

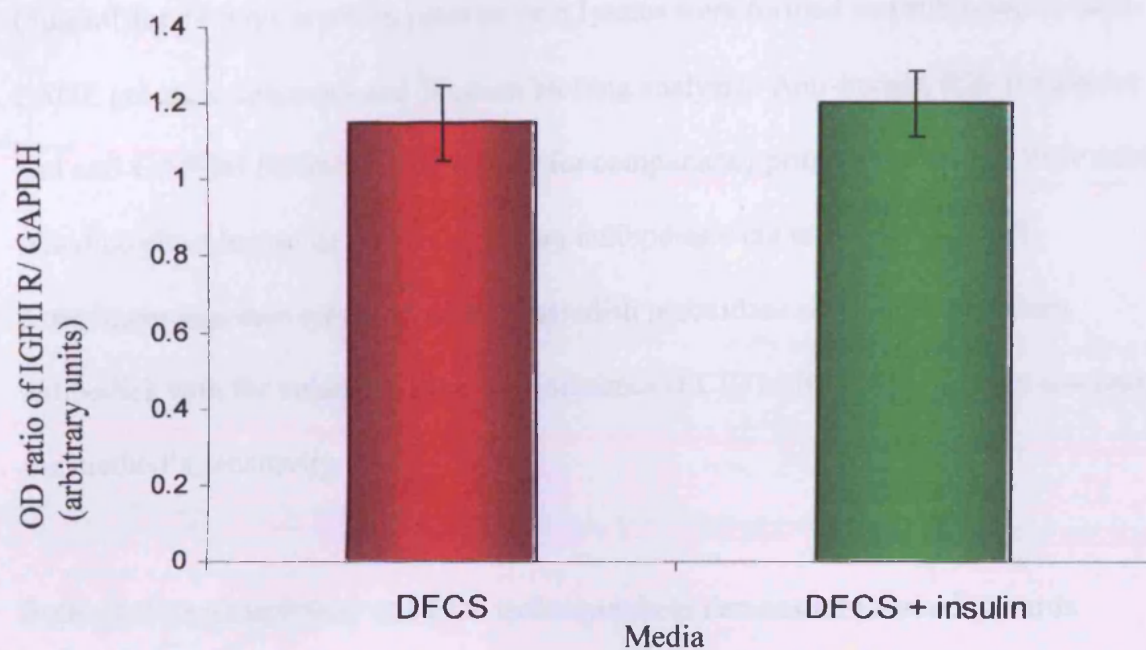


Fig. 5.6.3 OD ratio of IGF-II receptor / GAPDH mRNA of normal scar fibroblasts over 17 day time course. Media: DFCS with insulin (5 μ g/ml) (treated and without insulin (placebo). N=12 (untreated), N=7 (treated). No significant difference is seen between the two groups: Student t-test P=0.139. Error bars represent standard deviations from the mean.

IGF-II receptor protein expression

Normal scar fibroblasts were cultured in T75 flasks in DFCS with and without insulin (5µg/ml) for 14 days at which point protein lysates were formed and subjected to SDS-PAGE gel electrophoresis and Western blotting analysis. Anti-human IGF-II receptor and anti-GAPDH (housekeeping protein for comparison) primary antibodies were used. Alkaline phosphatase conjugate secondary antibodies were used initially. The experiment was then repeated using horseradish peroxidase conjugate secondary antibodies with the enhanced chemiluminescence (ECL) technique in order to increase the method's sensitivity.

Both alkaline phosphatase and ECL techniques both demonstrate a trend towards reduced protein expression of the IGF-II receptor in insulin treated fibroblasts although this does not reach statistical significance ($P=0.7$ and $P=0.47$ respectively) (see figs. 5.6.4 and 5.6.5), which may have been due to the high levels of variability between samples in the untreated group (see size of SEM bars).

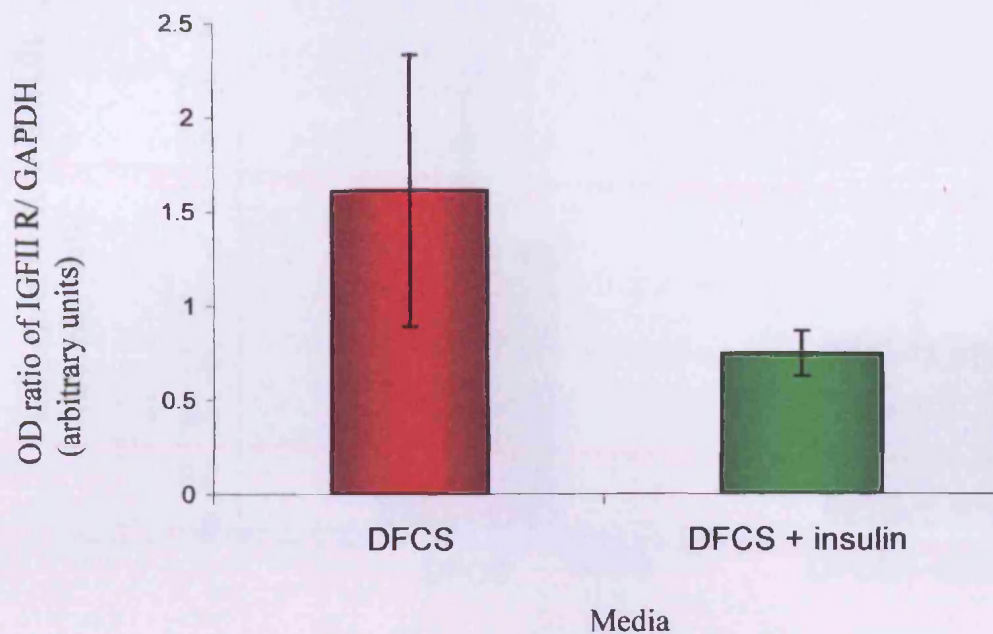
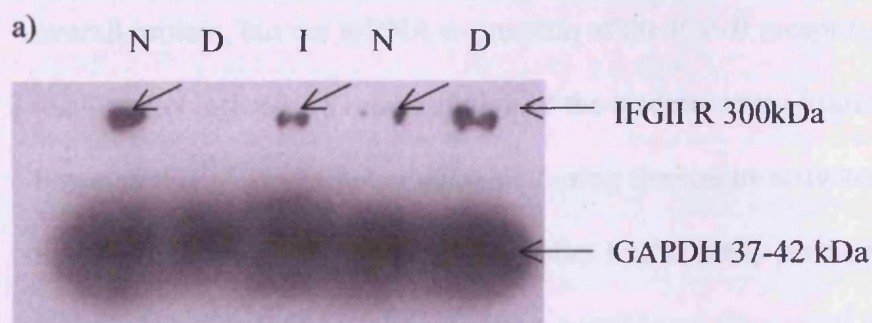
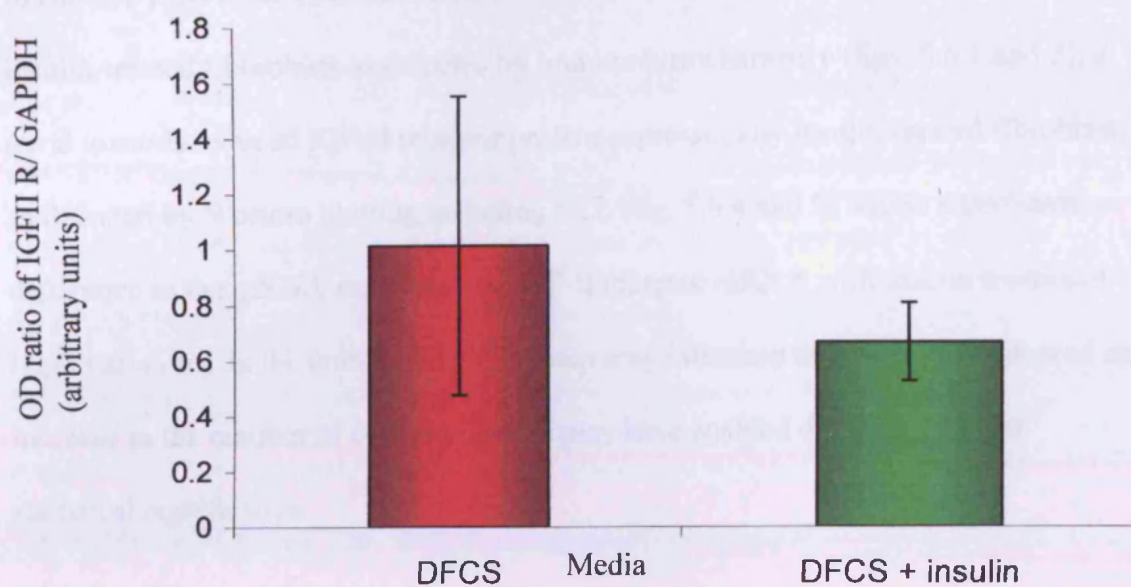


Figure 5.6.4 Densitometry analysis of western blots for the IGF-II receptor taken from normal scar fibroblasts cultured in 4% DFCS with and without insulin (5 μ g/ml) for 14 days. N = 3. Error bars represent SEM. Mann-Whitney Rank Sum Test, P = 0.700.



b) ECDL IGFII-R

Figs. 5.6.5 a and b. a) Densitometry analysis of ECL western blots for the IGFII receptor taken from normal scar fibroblasts cultured in DFCS with and without insulin (5 μ g/ml) for 14 days. N = 5 cell strains. Error bars represent SEM. Student t-test P=0.47. b) Representative ECL film (this film shows over-exposure of GAPDH, note GAPDH quantification was done on shorter exposed films). (N – NGM, D – DFCS, I – DFCS + insulin).

5.6.3 Discussion

In summary the results indicate a trend towards reduced numbers of IGF-II receptors on insulin-treated fibroblasts as detected by immunohistochemistry (figs. 5.6.1 and 2), a trend towards reduced IGF-II receptor protein expression by insulin-treated fibroblasts as detected by Western blotting including ECL (fig. 5.6.4 and 5) but no significant difference in the mRNA expression of IGF-II receptor mRNA with insulin treatment. High variability in the untreated DFCS group may influence this. Had time allowed an increase in the number of cell strains used may have enabled demonstration of statistical significance.

Interpreted together these results (fig. 5.6.1-5) suggest that insulin may reduce the overall protein, but not mRNA expression of the IGF-II receptor. It is still possible that insulin may influence a redistribution of the receptor away from the cell surface however this would be better examined using fluorescein-activated cell sorter analysis. If these conclusions prove to be founded after further study perhaps with higher numbers of cell strains then this would indicate a possible mechanism of action of insulin, that is, downregulation of IGF-II receptor protein expression with or without reduced cell surface localisation leading to depressed activation of latent autocrine TGF- β 1 and therefore lower levels of active TGF- β secreted by fibroblasts following insulin treatment as illustrated by section 5.4. Is such a mechanism plausible? Insulin is known to affect the synthesis of various proteins at either the mRNA transcription or protein translation stages for example vimentin, EH-domain containing protein 2, glucose

regulated protein 78 (GRP 78) and succinyl CoA transferase (Bluher et al. 2004). Thus it seems credible that insulin could affect the protein levels of the IGF-II receptor too.

There are precedents that support the possibility that insulin causes the redistribution of the IGF-II receptor. IGF-I, II and phorbol ester are known to increase the surface expression of the IGF-II receptors in a rapid mechanism independent of protein synthesis (Damke et al. 1992). The mechanism by which this occurs is unclear but IGF-I may induce the redistribution via intrinsic tyrosine kinase activity of the IGF-I receptor and IGF-II coupled with the IGF-II receptor to Gi2 proteins (Murayama et al. 1990), although further downstream effects of Gi2 are unknown. The significance of this redistribution is also unknown. More importantly, insulin has been shown to induce redistribution of glucose transporters and transferring alpha 2-macroglobulin and IGF-II receptors in human fibroblasts (Cushman et al 1980; Davis et al 1987; Wardzala et al 1984; Covera et al 1989)(Brault et al. 1989). Unfortunately, this redistribution was shown to be towards the cell surface rather than internally. Nevertheless, if insulin can increase the IGF-II receptor's distribution at the cell surface, maybe under different circumstances it can reduce it too.

Criticisms can be made of the methods used here. For example, the interpretation of the immunohistochemistry is problematic. Why some cells stain strongly, and some not at all is unclear since the IGF-II receptor is thought to be expressed ubiquitously. The judgement of 'positivity' is inevitably subjective and its significance unclear. Nevertheless, it helps to give a more complete picture of the situation.

That insulin could effect the protein expression or cell distribution of the IGF-II receptor would appear plausible although due to high variability the results (figs. 5.6.1 to 5) are inconclusive. However, the interaction between insulin and the IGF-II receptor need to be explored much further in terms of subcellular localisation studies, relative affinities for different ligands and the signalling pathway(s) or gene transcription/translation activation(s) that link insulin and the receptor. Such experiments are not feasible within the time frame of this research however but will be awaited with interest.

5.7 Discussion and Conclusion

The results of this chapter suggest that insulin may inhibit the differentiation of myofibroblasts by a similar mechanism to mannose-6-phosphate (statistically significant – see figs. 5.2.1 a-d), there is a trend towards higher levels of latent TGF- β 1 in insulin treated fibroblasts implying less activation (see figs. 5.3.1 – 7) and supported by the findings of significantly less active TGF- β in fibroblast cultures treated with insulin (figs. 5.4.1 and 2). Thus the possible mechanism of insulin's action does appear to be that it reduces the activation of autocrine latent TGF- β 1. The examination of whether this was via reduction of the IGF-II receptor was inconclusive. Further work using greater numbers of cell strains will hopefully clarify its role.

This mechanism has both strengths and weaknesses. The most obvious strength is that the effect of inhibiting the action of the IGF-II receptor has already been shown to reduce the activation of TGF- β 1. This effect is so potent that M-6-P added to rodent wounds has been demonstrated by Shah and Ferguson to improve the subsequent scarring (Ferguson et al 2004). This has even led to M-6-P being developed into an antiscarring therapy and it is currently undergoing clinical trials (Ferguson et al 2004). However, this is also a potential problem for insulin's antiscarring future – if these two molecules do both act via the IGF-II receptor, then it could be argued that insulin does not necessarily bring anything new to the table. Furthermore, the competitive inhibition mechanism of M-6-P might also be expected to be more efficacious than insulin which may act indirectly especially as insulin is known not to be a ligand of the IGF-II

receptor (Braulke et al. 1989). On the other hand, despite the results of section 5.2 (non-additive effects of insulin and M-6-P); it may be that in the clinical setting that the two potential therapies have greatest effect in combination. One significant advantage that insulin has over the M-6-P, however is that it only requires a brief administration period to attain long-term action that M-6-P only achieves with long-term administration.

Irrespective of whether insulin acts in a similar fashion to M-6-P, insulin does have one significant advantage in that to achieve the same effect insulin treatment is needed only for a short period whereas that of M-6-P is required long-term. A criticism of the possibility of the IGF-II receptor mechanism of insulin is that although insulin has been previously shown to cause redistribution of the IGF-II receptor (Braulke et al. 1989), this was to, rather than away, from the cell surface. However, this redistribution is associated with a decreased phosphorylation of the receptors at the cell membrane (Corvera et al. 1988) which could also affect receptor function. Furthermore, only 10-20 % of IGF-II receptors are at the cell surface, being in equilibrium with the rest which are on internal membranes (Braulke et al. 1987). These internal receptors may be just as important as the cell surface receptors, for example, changes in the receptor concentrations in the Golgi network may affect the secretion or retention of other endogenous receptor ligands such as IGF-II (Braulke et al. 1989).

The difficulty with the hypothesis that insulin inhibits activation of autocrine TGF- β 1 via the IGF-II receptor is that there are several other sources of activation available within the cell such as plasmin and other proteases. One potential alternative activator

of autocrine TGF- β 1 is thrombospondin, a glycoprotein produced by many cells (Crawford et al. 1998) that has been identified as a major endogenous activator of TGF- β in experimental inflammatory glomerular disease in rats (Daniel et al. 2004). Unfortunately attempts at immunostaining normal scar fibroblasts or producing Western blots for thrombospondin were unsuccessful. Nevertheless, it remains unclear whether such alternative mechanisms of TGF- β activation actually have physiological roles. The pronounced effect of M-6-P on myofibroblast differentiation testifies to the primacy of the IGF-II receptor as the activator of autocrine TGF- β 1.

The main criticism of these results must be that in many cases statistical significance has not been attained. However due to time limitations the numbers of cell strains tested were low (only 3) and so no judgement can be conclusively made as to the soundness of the hypothesis. Although individual experimental results were inconclusive, the trends exhibited by all (decreased active TGF- β 1/SLC ratio (figs. 5.3.1-7), together with significantly decreased active TGF- β levels (figs. 5.4.1-2) and a trend toward reduced IGF-II receptor expression/surface distribution (figs. 3.6.1-5) in insulin treated fibroblast cultures) point towards insulin reducing latent TGF- β activation.

This chapter began with an investigation into insulin's effects on α V β 5 integrin immunostaining of fibroblasts (see figs. 5.5.1 and 2) which suggested that α V β 5 integrin does not form part of insulin's mechanism of action, although much work is still required in this area with particular attention to α V β 5, α V β 1, α V β 6 and α V β 8. However, because there are a multitude of integrins with overlapping roles it seems

unlikely that insulin would exert its actions through inhibition of one integrin and yet it seems equally unlikely that insulin inhibits several integrins simultaneously.

Associated with the integrins are the metalloproteases, in particular MT1-MMP, a trans-membrane metalloprotease that has a role in α V β 8 mediated activation of TGF- β 1 LAP but also, itself activates TGF- β 1 LAP by proteolytic degradation at the cell surface (Mu et al., 2002). This was in fact examined during this study by SDS-PAGE gel electrophoresis and Western blotting; however, unfortunately, this did not yield meaningful results. Future studies would perhaps re-examine this protein.

This proposed mechanism of action for insulin is supported by the results of the previous chapters. That is, this mechanism is compatible with insulin not inhibiting other prerequisites to myofibroblast differentiation such as EDA-fibronectin (fig. 3.5.1-2), not preventing exogenous TGF- β inducing myofibroblasts (figs 3.4.1-2), not affecting the relative expression of TGF- β isoforms (chapter 4) and finally demonstrating significant association with reduced active TGF- β levels (figs. 5.4.1-2).

5.8 Conclusion

1. Transient exposure to insulin is as effective as continuous mannose-6-phosphate in inhibiting TGF- β 1-induced myofibroblast differentiation after the exogenous TGF- β 1 is removed (figs. 5.2.1).
2. The effects of insulin and mannose-6-phosphate are not additive suggesting that they work via a similar mechanism (figs. 5.2.1).
3. Insulin treated cells produce significantly less active TGF- β than untreated cells (figs. 5.4.1 and 2).
4. Insulin does not appear to affect the expression of the IGF-II receptor (figs. 5.6.1-5).

CHAPTER 6

**Prospective double-blind
randomised controlled trial of the
efficacy of insulin as an anti-
scarring agent**

6.1 Introduction

6.1.1 Rationale for trial

The in vitro and in vivo findings in this research so far suggest there is good reason to believe that insulin will inhibit scarring in patients. Although insulin's mechanism of action is still under investigation, its efficacy with regard to limiting the presence of myofibroblasts has been demonstrated in vitro (Linge et al, 2004) and in an animal model (Mackie 2004). The logical progression of this research is to test insulin's efficacy in a human clinical trial in parallel with continued exploration of its mechanism of action. Furthermore, the lag time between the discovery of a drug and its introduction into clinical practise is at least ten years even for the most powerful pharmaceutical companies. Therefore the sooner this phase of the research begins, the sooner patients will potentially benefit.

Short, medium, long and very long-acting commercially available human formulations of insulin have been tested in vitro at this laboratory so far. The long-acting insulin, Insulatard[®], proved the most efficacious in vitro and further more it proved effective in the murine model; with Insulatard[®] treated wounds showing approximately 60% less α -SMA staining (Mackie 2004). Therefore the evidence to support the use of Insulatard[®] in a clinical trial is stronger than for any other insulin formulation and it has been chosen for use in this clinical trial.

However, although the use of bovine insulin is possible in man, the existence of zoonoses such as Bovine Spongiform Encephalopathy (BSE), mean that any insulin tested in humans in a clinical trial would almost certainly have to be of a recombinant human variety. Bovine and human insulins differ by only 3 amino acids so their efficacies would be expected to be fairly similar and this has been demonstrated by Mackie (2004). The most efficacious of the human formulations of insulin previously tested by Mackie (2004) was Insulatard[®], a long-acting insulin (maximum effect 4-12 hours, see table 3.2). This chapter will examine whether the even longer-acting insulin, insulin glargine (no peak, 24 hours duration of action, see table 3.2), has a greater ability to inhibit the differentiation of myofibroblasts. The purpose of this investigation is to select the most effective recombinant human formulation of insulin for use in a clinical trial.

Irrespective of the outcome of this trial it will help with the future direction of this line of research. If the trial were to find that Insulatard[®] worsens scarring it would at least suggest that larger trials of Insulatard[®] should not be carried out. A null result would indicate that insulin is either not significantly effective in vivo or not effective at this dose. The trial will also inform future calculations regarding sample sizes and hopefully demonstrate safety. In short, whatever the result of the trial, it will alter the course of future research.

The trial will also give an opportunity to test methods of scar assessment, some new, prior to larger trials. Different assessment methods may be more suitable than others

depending on what sort of scarring Insulatard® affects be that normal or hypertrophic scarring. Other factors that may be specifically addressed in later trials would include feasibility, safety, dosage, formulation, efficacy in different patient groups, efficacy as part of combination therapies, efficacy on normal scars and efficacy on hypertrophic and keloid scars. This first trial therefore should be considered to be a pilot prior to larger trials addressing more specific issues.

However, there are arguments against undertaking the trial. Firstly, it is hoped that the investigation into insulin's mechanism of action reveals a pathway, receptor or signalling molecule whose manipulation could yield a more powerful and specific anti-scarring agent than insulin. Thus, the final drug may not be Insulatard® or any other insulin at all, but a different drug altogether making the findings from this trial less relevant. The counter argument to this is that if Insulatard® does prove to be effective then it could be introduced into practice very quickly as only a change of licence of use would be required. Insulatard® could thus potentially offer effective antiscarring treatment whilst the research yielded a definitive therapy.

Although the BALB/c mice that Mackie (2004) used for testing insulin in the animal wound healing model have been used extensively for such experiments (Stallmeyer, Kampfer et al., 2001), (Beer, Longaker et al., 1997), it could be argued that other animal models should be used before human trials as mice are known to scar well (Yang et al., 2007). However, human cutaneous scarring is notoriously difficult to mimic in animal models, excessive scarring such as hypertrophic scarring (the form of

scarring most of interest here) being present only in horses and recently the red Duroc pig (Zhu et al. 2003) and keloid scarring being absent in every other species. Therefore a repeat of the animal trial in a further species would not necessarily be beneficial. In addition, for participants in a human trial there is a very real possibility of benefit but with a very low chance of significant adverse events occurring.

In conclusion, the research so far supports the undertaking of a clinical trial using Insulatard® and its results will guide the direction of future research.

Aims

- To determine if the very long-acting recombinant human insulin, insulin glargine, is more effective at reducing fibroblast-myofibroblast differentiation than the long-acting insulin, Insulatard®.
- To demonstrate the efficacy (or not) of a human formulation of insulin in the reduction of postoperative scar severity.

6.2 The effect of the pharmacokinetic profiles of insulin formulations on their ability to inhibit myofibroblast differentiation

6.2.1 Introduction

The ideal antiscarring agent would be effective with a single dose applied within a short time of wounding . Insulin in the murine model appears to meet this criterion. By whatever mechanism insulin achieves this effect it must some how interact with the signals that either originate or control the long and complex chain of events that ultimately leads to scarring (Grazul-Bilska et al. 2003). Wounding sets in motion a whole host of signals and cascades over a period of days. Therefore, for a single dose to be effective in preventing scarring the drug concerned probably needs to be active over a period of hours and preferably days. There exists a range of commercially available formulations of insulin for the treatment of diabetic patients that vary with respect to their onset of activity and duration of action (British National Formulary, 2006) - see Table 6.2.1.

Name	Description	Time of onset	Maximum effect	Duration of action
Actrapid®	Short acting	30 minutes	1-3 hours	8 hours
Mixtard 30®	Intermediate acting	30 minutes	2-8 hours	24 hours
Insulatard®	Long acting	1.5 hours	4-12 hours	24 hours
Insulin Glargine (Lantus®)	Very long acting	2 hours	No peak	24 hours

Table 6.2.1 Table showing characteristics of insulin preparations chosen for Investigation (Manufacturers data – Novo nor disk and Adventism).

A selection of these formulations were tested by Mackie (2004) and showed that the longest acting formulation, Insulatard®, was the most effective in inhibiting myofibroblast differentiation in vitro whereas surprisingly Actrapid had a significantly smaller effect. Comparison of the pharmacokinetic profiles of these two formulations therefore suggests that a delayed peak might be important in eliciting the myofibroblast inhibitory effect. Insulatard's® efficacy was further confirmed in a murine in vivo model that demonstrated significant inhibition of myofibroblast numbers in the scars of incisional and excisional wounds treated with Insulatard® (Mackie 2004). Yet there exist insulin formulations with an even longer duration of action. Such formulations may continue the trend of greater efficacy with longer durations of action. Therefore the efficacy of Insulatard® was compared with the longer acting insulin glargine with the intention of identifying the most effective agent for the subsequent clinical trial. Insulatard® lasts for 24 hours but reaches its peak activity after approximately 8 hours and declines thereafter, In contrast, insulin glargine produces constant insulin plasma levels for 24 hours without a discernible peak. Thus after 12 hours plasma insulin levels

are higher with insulin glargine following equivalent doses of the two formulations. Therefore, insulin glargine may prove more effective than Insulatard®. This experiment seeks to test this and in so doing aid the selection of the formulation to be used in the clinical trial.

6.2.2 Method

Insulatard® and insulin glargine were tested for their ability to reduce fibroblast - myofibroblast differentiation. Three normal scar fibroblast cell lines were cultured on sterile glass coverslips in triplicate in NGM, DTTM or DTTM supplemented with insulin. The concentrations of commercial insulin formulations are described in terms of International Unit (IU); a concentration of human insulin of 1 IU/ml is equivalent to supplementing medium with bovine insulin at 50 µg/ml. A titration range of 0.01, 0.05, 0.1, 0.5 and 1.0 IU/ml was derived from this conversion and applied to each formulation. After 14 days treatment the proportion of myofibroblasts present was assessed following immunohistochemical staining to demonstrate the presence of α -SMA (see Section 2.4). The two insulins were not used beyond their post opening expiry date.

6.2.3 Results

Comparing the myofibroblast inhibiting efficacies of the two formulations (Figure 6.2.1) demonstrates that both formulations inhibit myofibroblast differentiation at even the lowest concentrations. However, significance is only achieved at concentrations of

0.5 IU/ml (Insulatard® - 7% myofibroblasts, $P<0.01$) and 1.0 IU/ml (insulin glargine – 9% myofibroblasts, $P<0.05$). At these concentrations they reduce the proportion of myofibroblasts from approximately 28% in the DTTM control media to similar levels to that seen in NGM (5-10%).

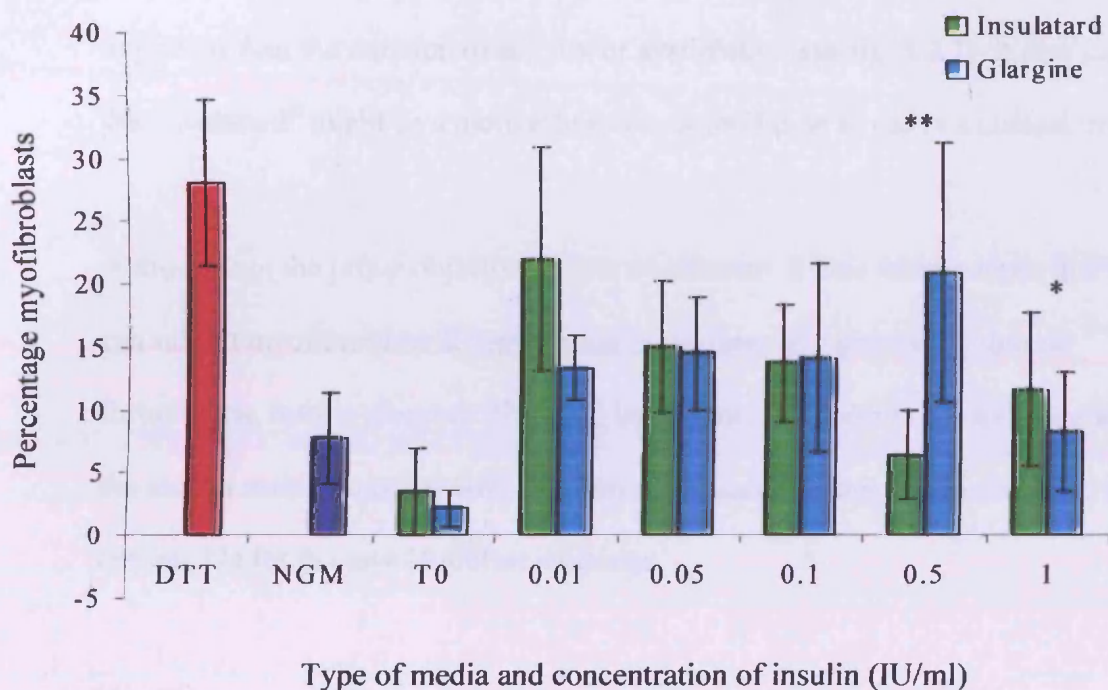


Fig. 6.2.1 Normal scar fibroblasts cultured on coverslips for 14 days in NGM and DTTM alone or DTTM supplemented with a range of concentrations of long acting insulin (Insulatard®) and longer acting (glargine) insulin. Proportion of myofibroblasts expressed as percentages. N=3 normal scar cell strains. Statistical comparison is with the control: DTTM. Insulatard® shows significant myofibroblast inhibition at 0.5 IU/ml, Student t-test $P < 0.01$ (**); insulin glargine shows significant myofibroblast inhibition at 1.0 IU/ml, $P < 0.05$ (*).

6.2.4 Discussion

Both formulations of insulin demonstrate efficacy in the inhibition of myofibroblast differentiation with Insulatard® showing significant inhibition at a lower dose than insulin glargine suggesting that the peak of insulin activity or availability is more important than the duration of activity or availability (see fig. 6.2.1). It also suggests that Insulatard® might be a more efficacious formulation to use in a clinical trial.

Although not the prime objective of this experiment, it also demonstrates that insulin can inhibit myofibroblast differentiation in the form of a previously untried formulation, insulin glargine. This thus lends further support to the assertion that it is the insulin molecule rather than an additive particular to any one formulation that is responsible for the myofibroblast inhibition.

6.3 A prospective randomised clinical trial to determine the efficacy of insulin as an antiscarring agent

6.3.1 Choice of Procedure

Scarring therapies have been tested in numerous scenarios. Scars in various anatomical sites have been used in trials for example, small incisions on the inside of volunteers' arms (Ferguson and O'Kane, 2004), facial acne scars (Chua et al. 2004), burn scars on limbs (Kealey et al. 1990) and surgical scars on the abdomen (Atkinson et al, 2005). Each of the different types of scar have also been used – keloids (Har-Shai et al. 2006); Berman & Bielely, 1996), hypertrophic scars (Saray et al. 2005); Atkinson et al, 2005), normal scars (Laccourreye et al. 2005); Berman et al, 2005) and atrophic scars (Friedman et al. 2004) (Chua et al, 2004).

The ideal clinical trial is one in which the patients acts as their own controls by having two separate wounds on each side of the body in identical locations. These should be wounds incised for the same purpose so that the level of tissue trauma is the same and thus the stimulus to scar formation is similar. The wounds should be closed in the same way by the same surgeon or surgeons of very similar experience. In addition, to maximise the chance of detecting improvement in scars, the wounds should be at a site that is prone to hypertrophic scarring but also inconspicuous enough that asymmetry between the scars doesn't cause patient dissatisfaction. The procedure also needs to be performed frequently and electively so recruitment of enough patients is both possible and feasible. The patients should be healthy so as to avoid the uncontrollable effects on healing and scarring that systemic disease may have.

Although procedures for trauma are performed frequently the level of tissue trauma is widely variable and symmetrical wounds are rarely provided. Elective skin cancer excisions are also undertaken frequently and would provide a breadth of anatomical sites, but again lack symmetry. An alternative to using two symmetrical incisions would be to divide each incision into control and placebo lengths, perhaps separated by an intermediate no treatment/placebo stretch. This would be feasible for large incisions in elective surgery. Skin cancer excisions tend to produce wounds of 5cm or less. At this length the diffusion of insulin (or its signals) into the placebo length would be difficult to argue against. Trauma wounds are simply too uncontrolled for one length to be fairly compared to another.

These considerations exclude the majority of plastic surgical procedures but bilateral breast reduction surgery does fulfil these criteria. This is a common, elective procedure to reduce the weight of the breasts and simultaneously improve shape. It involves an anchor-shaped incision that circles the areola, extends downward, and follows the natural curve of the crease beneath the breast. After breast reduction the line of incision, lying directly below the breast, often develops hypertrophic scarring at the medial, and to a lesser degree, the lateral ends. This is thought to be related to the weight of the breasts causing maximum tension in these parts of the wound. Although patient satisfaction surveys following breast reduction surgery have repeatedly demonstrated high satisfaction with the procedure, the inframammary (crease between the breast and chest wall) scar remains the greatest source of dissatisfaction in

particular because of its poor colour match with the surrounding skin (Celebiler et al. 2005).

The usefulness of breast reduction surgery for testing antiscarring therapies has been exploited by other authors. For example, Bellew et al in 2005 (10 patients) compared the effects on the whole length of breast reduction scars of long-pulsed dye lasers and intense pulsed light lasers showing reduction in purpura of the scars (Bellew et al. 2005); Niessen et al in 1997 showed improved inframammary scarring with the use of single compared to multiple filament sutures (Niessen et al. 1997) (81 patients), and Cruz-Korchin demonstrated reduced rates of hypertrophic scarring over the whole breast incision with the postoperative application of silicone gel sheets for 2 months (20 patients) (Cruz-Korchin 1996).

The length of scar breast reduction surgery produces is approximately 40cm per breast. Infiltrating insulin into this length of wound would be time consuming, could lead to a metabolically significant insulin dosage and is in any case not necessary for the trial. Therefore a short portion of the inframmary scar will be chosen for insulin injection. For consistency of location either the medial or lateral end of the scar would be preferable. The medial end of the scar encroaches on the sternum and is very visible; the lateral end of the scar encroaches into the axilla. Therefore any asymmetry that results will be less noticeable if the lateral ends of the scars are chosen. Although wounds in the region of the sternum are notorious for their tendency to hypertrophic scarring, the rate of hypertrophic scar formation in all parts of the untreated

inframammary wounds range from 8.6% (), 39% (Lewis et al. 1990) to as high as 60% (Cruz-Korchin 1996) and 65% (Niessen et al. 1998). Therefore the lateralmost portion of the inframammary scars will be chosen for the insulin/placebo infiltration.

In summary, the choice of breast reduction surgery for this trial has the advantages of two separate incisions in identical anatomical sites, a known hypertrophic scarring rate, it's an elective procedure performed on usually healthy patients and it's been previously used in trials of antiscarring therapies and techniques.

6.3.2 Safety concerns

Insulatard® has an excellent safety record with no reported incidences of anaphylaxis or any other serious adverse effects (Chiou et al. 2005). Any insulin has the potential to be lethal in massive overdoses, however, in this study very small doses (0.3 IU) are administered to patients compared with the doses routinely administered to diabetic patients (4 to over 50 IU) and dose checking involving at least two people minimises the risk of erroneous dosing. 0.3 IU is simply too low a dose to have any discernible hypoglycaemic effects and this has been confirmed by discussions with a Consultant Endocrinologist. Indeed, Mackie (2004) administered 0.3 IU to mice with no ill effects. Possible side-effects are local to the site of infiltration such as skin irritation, swelling, pain and bleeding. All of these would be expected to be minor and to have resolved by the time the patient has recovered from general anaesthetic.

6.3.3 Methods of scar assessment

With the exception of the Manchester scar scale and panel assessment of digital photos of the scars, the methods of scar assessment described here are novel. This is for two reasons. Firstly, there is no objective, widely accepted and available tool for reliable scar measurement. Secondly, the methods used are inexpensive and readily available at this laboratory. In contrast, other scar measurement devices, which are not in clinical use, are difficult to obtain and expensive.

Histopathological examination is the gold standard for scar assessment but is not appropriate in many clinical scenarios, thus clinical tools have been developed that facilitate objective assessment of scars. The first, the Vancouver Burn Scar Assessment Scale, rates scars on pigmentation, vascularity, pliability and height. (Sullivan et al. 1990) This initial concept has been further developed to make descriptions numerical; to include scar location and patient observation, and to broaden the application to linear non-burn scars. (Masters et al. 2005), (Draaijers et al. 2004), (Beausang et al. 1998)

There are several scar scales published in the literature to choose from. This study required a scale that can be applied to linear surgical scars, shows good interobserver correlation, easy to understand, correlates with the histological appearances and has been properly validated. The Manchester scar scale fulfils all of these criteria and so was the one chosen.

The clinical scar scale and panel assessment is at the core of the scar assessment in this trial. The additional methods really serve to corroborate or challenge the findings from the panel assessment but in addition provide objective measurements to compensate for the inevitable degree of subjectivity incorporated into the use of clinical scar scales.

Scar scales have the advantage that they allow for the simultaneous assessment of several of the key characteristics of a scar that make it symptomatic but in addition rates the overall cosmetic impact that a scar has which is one of the most important consequences of a scar but one that is hard to quantify technologically.

3-D digital imaging techniques that allow accurate calculation of scar volumes would be ideal for this trial, however, they are currently experimental and at present are also inaccurate at calculating the very low volumes that many of the final scars in this study represent (Rawlins et al. 2006). Other technologies measure redness/erythema (e.g. Minolta Chromameter), pigmentation (e.g. DermaSpectrometer®), thickness (ultrasound), surface area and texture (digital photography and optical or mechanical profilometers) and suppleness (e.g. Cutometer®) (Niessen et al. 1998), (van Zuijlen et al. 2002), (Draaijers et al. 2004).

Several different techniques have been chosen in order to maximise detecting any effect on scar formation that insulin may have. Thus there is a technique for measuring each main characteristic of a scar, namely height (multiphoton microscopy), width (digital image analysis), volume (silicone moulds), scar colour (digital image analysis) and shininess (digital image analysis). Because the objective assessment of scars is so

challenging and open to question it is hoped that by measuring as many aspects of the scars as possible in this study, the conclusions drawn will be more credible. Methods of scar assessment are discussed in greater detail in the introduction to the thesis (see section 1.7.2)

6.3.4 Ethics committee approval

Prior to commencement approval of the study was obtained from the East Hertfordshire Ethics Committee and the Medicines and Healthcare Products Regulatory Agency (MHRA). The trial was also registered with the European Database of Clinical Trials (EuDraCT).

The main issue of contention was the possibility of insulin being very effective and thus giving patients significant asymmetry of their scars. This was a real possibility but as scars naturally fade and flatten over time the asymmetry would be temporary. It also did not seem likely that the insulin would induce completely scarless healing. Finally, there is inevitably some degree of asymmetry following this surgery which reflects the asymmetry of the breasts preoperatively.

6.4 Materials and methods

6.4.1 Estimation of sample size

The estimation of sample size is to some degree unavoidably arbitrary as without any previous clinical trials or even anecdotal evidence there is no way of knowing if insulin would work, by how much and in what proportion of patients. Another major unknown is whether insulin would effect normal scars or whether it would only effect hypertrophic scars. This is important because although we can guarantee at least normal scar formation in all of the participants, the incidence of hypertrophic scarring varies wildly between different case series (see section 6.1.2). However, a relatively high rate of hypertrophy (in the untreated scars) would be desirable because demonstration of difference is easier if the treated and untreated scars occupy opposite ends of the spectrum.

The actual sample size of 15 patients was determined by a professional statistician at the University of Hertfordshire using paired sample designs to advise on the number of patients. It was based on the range of Manchester scar scale scores that it was expected would be seen (5 to 18 or more out of 28) and not on changes in scar prominence, volume or any of the other objective measures. To make the estimate two predictions have to be made regarding the degree to which insulin improved scars and the proportion of scars we would expect to be hypertrophic. For insulin to be worth pursuing as antiscarring agent, it must have a visibly noticeable effect. Thus an

improvement of 5 points in the Manchester scar scale score of a treated scar compared to control scar was chosen.

The second assumption is that 30% of the scars would be hypertrophic. This is a conservative figure compared with the rates quoted in the literature (see section 6.1.2) but has been arrived after discussing the experiences of surgeons at Mount Vernon Hospital.

The sample size was also limited by practicalities. Although enrolling a greater number of patients in the study would be desirable, in practise it would have proved difficult to achieve due to the limited number of patients undergoing this procedure but also because of the logistical problems of arranging for the same surgeons to do all of the procedures.

6.4.2 Patient Recruitment

Patients on the waiting list for bilateral breast reduction surgery at Mount Vernon Hospital were initially approached by a telephone call from the investigator followed by a letter and patient information sheet (see appendix II). On the day before surgery patients were seen on the ward and given the opportunity to ask more questions and if they were in agreement, written consent was obtained. It was stressed to the patients that their participation was voluntary and if they declined to take part in the study this would in no way prejudice their treatment.

6.4.3 Exclusion criteria

The exclusion criteria were as follows:

1. Younger than 18 or older than 60- (children and adolescents are known to be more susceptible to developing hypertrophic scars whereas the elderly tend to form fine, inconspicuous scars (Crikelair 1960).
2. Non-Caucasian (because of the different scarring tendencies of people of Asian and Afro-Caribbean descent - i.e. greater tendency to keloid scarring (Alhady et al. 1969).
3. Patients with a history of hypertrophic or keloid scarring.
4. Smokers - impairs wound healing (Forrest et al, 1987).
5. Any systemic illness that could have a theoretical interaction with the insulin administered such as diabetics, patients with renal or liver disease or endocrine tumours.

The exclusion criteria are chosen to make the patient group as homogeneous as possible in terms of their likely propensity toward abnormal scarring. A patient with a history of keloid scarring for example, could potentially skew the overall results so much that any effects in the other patients are masked. Likewise, an elderly patient could similarly skew the results by healing with very little scarring regardless of treatment. In addition, it could be argued that the aetiology of keloid and hypertrophic scars are so different that they cannot be properly examined together in a single trial.

In practise these criteria exclude very few possible participants as patients who do fit any of these criteria are unlikely to be put forward for breast reduction surgery anyway. Only 1 patient was recruited and subsequently rejected on the basis of having a skin type that could predispose her to keloid scarring.

6.4.4 Randomisation method

Fifteen pieces of paper were prepared; eight had “syringe with suture right breast, syringe without suture left breast” written on them and the other seven had “syringe with suture left breast, syringe without suture right breast” written on them. The “syringe with suture” refers to the syringe containing the dilute insulin solution and the “syringe without suture” refers to the syringe containing saline placebo (see section 6.3.5) These pieces of paper were each glued into the inside of envelopes which were sealed with paperclips and then shuffled and put into a basket. For each patient one of the envelopes was randomly selected by a co-worker unrelated to the project. Before surgery the surgeon was given the envelope and asked to look inside and memorise the instructions. After the surgeon, and only the surgeon, had seen the contents, the envelope was sealed and a patient sticker affixed to the front (fig. 6.4.1). The surgeon was not told the significance of the suture (marking the syringes containing Insulatard[®]). After the surgery the surgeon made a note in the operation record specifying which breast had been injected with which syringe. All of the envelopes were stored securely until the trial was finished and all the results had been analysed. The study was unblinded by opening the envelopes and recording which breasts received the treatment and placebo. This was cross-checked with the operation records.

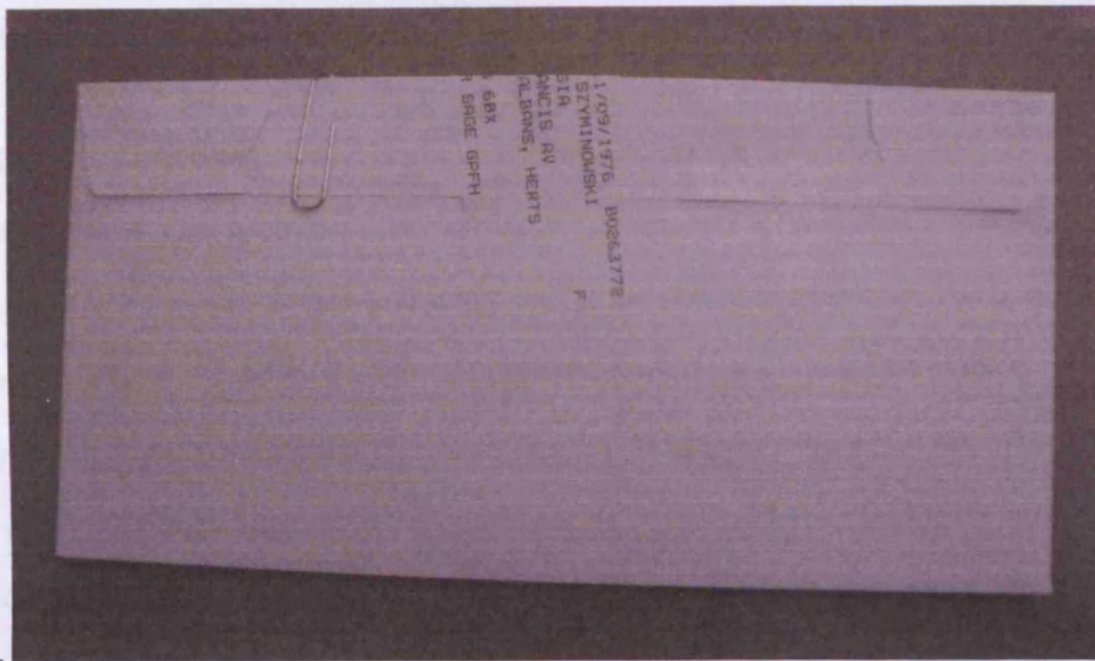


Fig. 6.4.1 Envelope containing instructions for surgeon sealed with a patient-identifying sticker. This remained sealed until after analysis of the results.

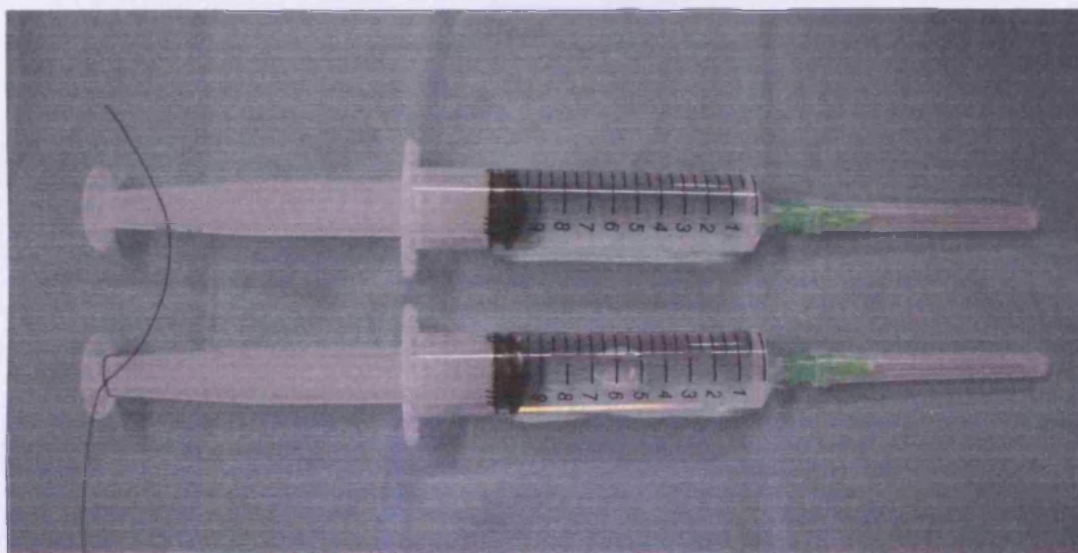


Fig. 6.4.2 Two 10 ml syringes one containing 1.0IU Insulatard® (marked by the suture) and the other containing solely saline. Note that the liquids are indistinguishable in appearance.

6.4.5 Drug administration

The formulation of insulin that was chosen was Insulatard[®] because this has demonstrated the greatest efficacy in vitro compared with other human formulations of insulin also because it was successfully used in the animal experiments. The dose administered was 0.3 IU for each 3cm of test wound. This is equivalent to the 0.15 IU per 1.5cm wound administered in the murine model. The efficacy of insulin in the in vitro experiments suggests that insulin exerts its antifibrotic effect at a 'local' rather than systemic level; therefore the dosage hasn't been multiplied according to the difference in human/mouse body masses.

A trial involving multiple different doses of Insulatard[®] would clearly be desirable as there are so many factors that could affect the optimum dose in the patient.

However, this would require many more participants; therefore this trial will use the dosage for which there is most evidence.

Although transdermal iontophoresis of insulin is possible (Pillai et al. 2003), it is still in the experimental stage so needle infiltration was chosen as the method of drug delivery. It is also cheap, requiring only syringes and needles that are widely available in operating theatres and is clearly the easiest way to administer the drug to wound edges at the end of an operation. 10 ml vials of 100IU/ml Insulatard[®] were purchased from the manufacturer and refrigerated until use and then discarded 6 weeks after first use as per the manufacturer's instructions.

During the surgery two 10ml syringes were prepared. One syringe contained 10mls of 0.9% normal saline. The other syringe contained 10mls of 0.9% normal saline plus 1 IU of Insulatard® (colourless at this concentration) and to this syringe was tied a suture. The surgeon did not witness the preparation of the syringes and was not told whether the suture marked the treatment or placebo. At the time of wound closure the surgeon infiltrated 1.5mls of solution to the dermis on each side of the wound (total of 3mls per wound) according to the instructions contained in the envelope. This meant a dose of 0.3 IU Insulatard® was administered to 3cm of wound on the test side. The surgeon recorded in the operation note which breast wound he had injected with which syringe. The author was not present when the wounds were infiltrated. In this way, neither the patient, surgeon, nor researcher knew which breast had received insulin until the trial was unblinded after the analysis of results.

Every patient had a single intraoperative dose of intravenous antibiotics and every wound was closed with dermal 4/0 Monocryl® and 5/0 subcuticular Prolene® and a suction drain (a tube draining blood and fluid from the wound – a routine surgical practice) which was removed once the drainage was less than 30mls/24 hours. The wounds were all dressed in the same way, namely: adhesive tape, gauze and adhesive foam (Microfoam®). Patients were discharged between 24 and 36 hours post surgery.

6.4.6 Patient follow-up and scar assessment

All patients were seen initially 1 week post surgery for removal of the nipple sutures. They were seen again 2 weeks post surgery for removal of the subcuticular Prolene sutures from the 'T' portion of the wound. Once the stitches were removed clinical assessment of the scars using the Manchester scar scale (fig. 6.4.3) was performed by the author and digital photographs taken of the relevant sections of scars.

All patients routinely had outpatient appointments 3 months post surgery and at this time clinical assessment of the scars was repeated, further digital photographs were taken and in addition silicone moulds of the scars were made.

Patient details			
Left	or	Right breast scar?	
VAS			
Poor		Excellent	
A	Colour (cf. to surrounding skin) Lighter or darker?	Perfect	1
		Slight mismatch	2
		Obvious mismatch	3
		Gross mismatch	4
B	Matte(1) or Shiny(2)?		
C	Contour	Flush with surrounding skin	1
		Slightly proud/indented	2
		Hypertrophic	3
		Keloid	4
D	Distortion	None	1
		Mild	2
		Moderate	3
		Severe	4
E	Texture	Normal	1
		Just palpable	2
		Firm	3
		Hard	4
Score:		/28	

Fig. 6.4.3 The Manchester scar scale. This scale was used for the clinical assessment of scars. A modified version that omitted the texture assessment was used for the panel assessment because texture cannot be determined from photographs.

6.4.7 Estimation of scar prominence and volume

Making silicone moulds of the scars

At the 3 month follow-up appointment silicone moulds of the scars were taken. The silicone was dental grade and obtained from the Mount Vernon Hospital Oral and Maxillofacial Surgery Prosthetics Department. The scars and surrounding skin were first cleaned and a thin film of petroleum jelly applied to prevent the mould sticking to hairs and becoming painful to remove. The silicone was then applied to the scars in an approximately 0.5x 3 x 8cm (depth x width x length) layer. The silicone comes in the form of two liquids which when mixed solidified within 10 minutes. The excess silicone was then trimmed from the moulds and then they were glued to glass slides for examination under the multiphoton microscope and for measuring the volume of the scars (fig. 6.4.4).

Multiphoton microscopy

Multiphoton microscopy is a technique that allows three dimensional reconstructions from two-dimensional images of structures smaller than one mm. It is based on the finding that two low energy photons can excite a fluorophore in a quantum event, resulting in the emission of fluorescence, so-called two-photon excitation. The silicone moulds were given a fluorescent hue by running a fluorescent marker over the relevant areas. The microscope scans the surface of the moulds and a computer records and interprets the myriad fluorescent emissions as a three-dimensional image. As the mould is scanned a changing image is displayed on the computer screen along with measurements recording depth. Thus the depths of the trough of the mould and the

highest point of the side-wall of the trough can be recorded and the first subtracted from the last to give an estimate of the depth of the trough in the mould which equates to the height of the scar.

Use of the microscope was kindly given by the Gray Cancer Institute at Mount Vernon Hospital.

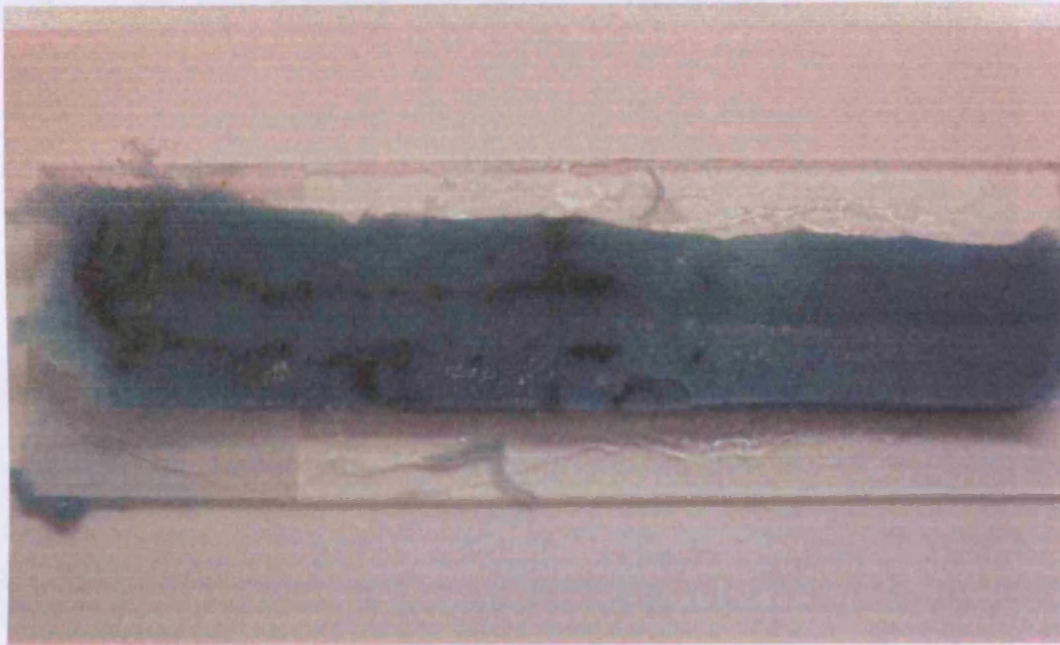


Fig. 6.4.4. Silicone mould of breast scar. Black ink outlines the margin of the scar. Fluorescent ink (difficult to see on the photograph) marks the 1cm, 2cm and 4cm distances from the lateral end of the scar at which the depth of the scar impression (scar prominence) was measured by multiphoton microscopy.



Fig. 6.4.5 The multiphoton microscope.

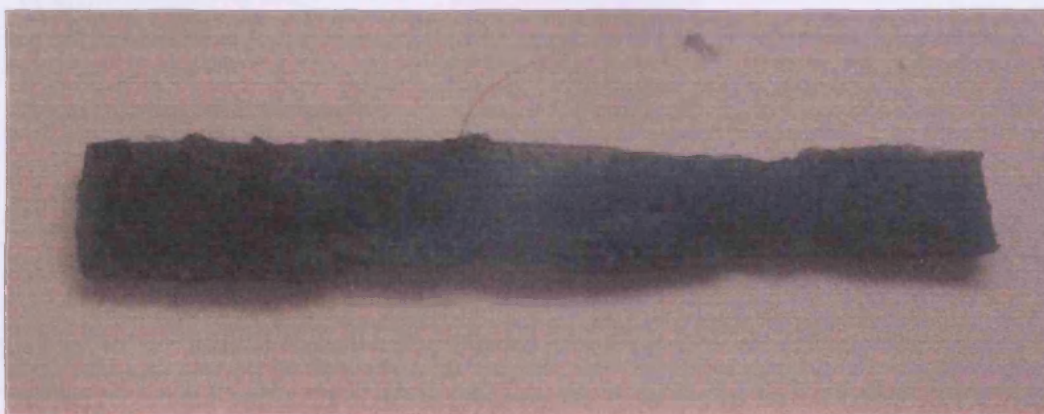


Fig. 6.4.6 An example of an inverted silicone mould taken from the mould of a breast scar.

Measuring scar prominence

The depth of the scar imprint was measured at 1cm, 2cm and 4cm from the lateralmost end of the scar imprint. The first two measurements are within the treated area and the last is outside the treated area. The first two measurements are averaged to give a mean value for the scar prominence and also compared to the prominence of scar in the untreated area and then compared with the same values from the other breast of the same patient. In this way, comparison can be made not just between the insulin-treated and placebo-treated regions of scar but also between the ratio of scar prominence between the test length of the scar and the untreated scar on the same side. This is to try and control for the inevitable variation between scars even in the same patient.

Three moulds representing the range of scar sizes were each measured three times by this method to give an idea of the reproducibility of the technique and this demonstrated a variation between readings of the same measurement ranging from 0 to 11%.

Determining scar volumes

This was achieved by making 'moulds of the moulds'. The silicone moulds that had been taken from the patients were coated with a thin film of oil (WD-40) and then liquid silicone applied to the surface of the moulds taking care to ensure the liquid filled the furrows that the scars had made. Whilst still liquid the excess silicone was removed from the moulds by dragging a glass slide over the surface of the moulds. A further glass slide was then pressed onto the mould until flush with the surface of the original mould to ensure no silicone was included beyond that contained in the trough. Once dry

the new mould was carefully removed with forceps, the oil preventing adherence to the original mould. Excess material was trimmed from the samples and they were then weighed and their volumes calculated from using the value determined for the solid silicone's density.

The volumes of three scars representing the range of scar sizes were each calculated three times by this technique which showed remarkable reproducibility with no volume estimate of a scar varying more than 5% from subsequent volume estimates of the same scar.

6.4.8 Digital photography and digital image analysis of scar colour, luminosity and width

At the 2 weeks and three month appointments digital photos were taken of each of the scars with the same camera (Casio EOS1N with Kodak DCS 520 digital back) by the same professional medical illustrator under the same lighting conditions. The same room was used and the camera was held from approximately 20 cm from the scar each time. At the time of taking the photos an adhesive metric scale was applied to the skin inferior to the scar and included in the image.

The width of the scars was determined from the digital images by using the metric scale to measure the scar widths at 1cm, 2cm and 4cm from the lateralmost end of the scar. The images were then imported into PowerPoint and divided into three 10mm x 2mm blocks. These images, complete with blocks were then imported into Adobe Photoshop (see fig. 6.4.7).

A further rectangle of almost the same size was then outlined within each of the blocks ensuring the number of pixels remained the same for each of these rectangles. The luminosity, redness and greenness (arbitrary units) and number of pixels were recorded for each block using Adobe Photoshop. To control for variation in the pigmentation and luminosity in the skin of patients, the same measurements were taken from three contiguous 10mm x 2mm blocks of skin inferior to each scar. The measurements from each section of scar were compared with the corresponding sections of skin. The

resulting values were then compared with the same values obtained from the opposite breast of the same patient.

Redness and luminosity in this context are indirect measures of inflammation or ongoing scarring activity which would be expected to be increased in cosmetically worse hypertrophic scars. Their importance in the overall appearance of a scar is reflected in their inclusion in clinical scar scales but also in the scar assessment technologies that measure them, particularly redness.

Provided the rectangles outlined on the Photoshop images were of the same size (number of pixels), repeated measurements of red, green and luminosity gave approximately the same values with a spread of less than 1%.

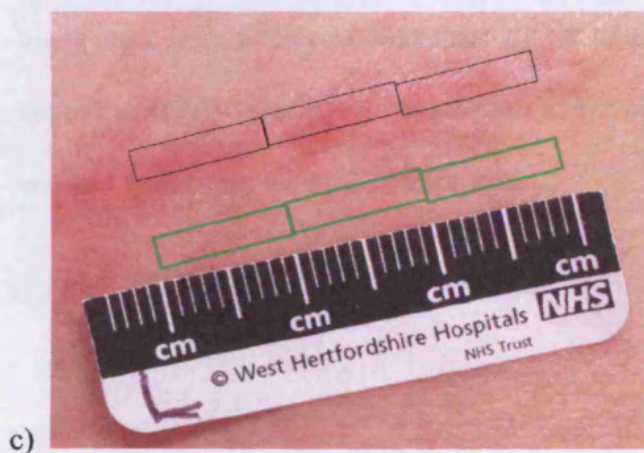
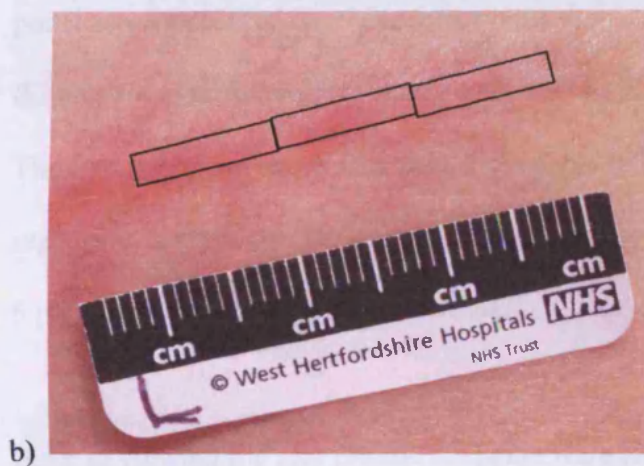


Fig. 6.4.7 a, b and c. Digital image analysis of the scars. (a) Digital images of the scars are imported into Photoshop[®] making sure the images are of the same size and number of pixels. (b) The scar is divided into three 10 x 2 mm blocks and the red, green and luminosity measures (arbitrary units) are recorded using Paintshop Pro[®]. (c) Another three blocks of the same dimensions highlighting adjacent skin are then analysed in the same way to give intrapatient same-side controls. The resulting ratios are compared with the ratios obtained from the image of the scar on the other breast to give a final placebo-treatment comparison.

6.4.9 Panel assessment

Panel assessment refers to the practise of having a ‘panel’ of several people to assess, in this case, digital photographs of the scars. The aim is to generate an objective assessment of individual scars from multiple subjective impressions.

The panel assessment was prepared as a PowerPoint presentation and delivered to two separate groups. The digital images of the scars were each given a number and then put in a random sequence determined by a random number generator. For each image the panel assessors filled in a Manchester scar scale. The section for scoring the profiles of the scars was omitted as this cannot be reliably inferred from a 2-dimensional image. The first group consisted of 8 plastic surgeons (4 senior house officers, 4 specialist registrars) and the second group was mixed consisting of 8 scientists, 4 lay people and 5 plastic surgeons (senior house officers).

Prior to viewing the scar photos the panel were shown examples of an excellent scar, poor scar, hypertrophic scar and keloid scar with their corresponding scores to help make more informed decisions regarding the scores of the test scars. They were given approximately 30 seconds to assess each image and assessments had to be made without conferring with other assessors.

6.5 Results

6.5.1 Patient Recruitment

Nineteen patients were approached and invited to take part in the trial, sixteen accepted although one was excluded on the basis of having significantly darker skin than the other patients which would make digital image assessments harder to compare with the other patients and would also possibly increase the risk of subsequent keloid scarring. The ages of the fifteen patients ranged from 21 to 54 years with an average age of 36 years. They were all fair-skinned, healthy non-smokers with no history of pathological scarring.

6.5.2 Complications

One patient developed a postoperative wound dehiscence and subsequent wound infection at the junction of the vertical and horizontal limbs of the scar on one breast (placebo side). This infection resolved with antibiotics. No patients developed symptomatic hypoglycaemia.

6.5.3 Scar volumes

The total difference in volume between all the treated and untreated scars is: +52.0 μl (i.e. treated group total higher volume). There was no significant difference in volumes between the two groups. However, if the scars are compared on a patient by patient

basis, in 9 out of 15 patients the volume of scar was greater in the untreated scars (see fig. 6.5.1). In addition, of the 6 patients who showed a larger scar on the insulin-treated side, in two of the patients (11 and 12) exhibited very large differences in volume which may have skewed these results. When the data is expressed as percentage difference in volume between insulin- and placebo-treated scars of each patient (see fig. 6.5.2), in 9 out of 15 patients the untreated scar is bigger. If the percentages difference for each patient is summed the overall finding is that the placebo-treated scars are larger in volume with each individual having an placebo-treated scar on average 15.4% bigger than their insulin-treated scar. Thus, although statistical significance has not been reached, there is a trend towards lower volumes in the insulin treated scars.

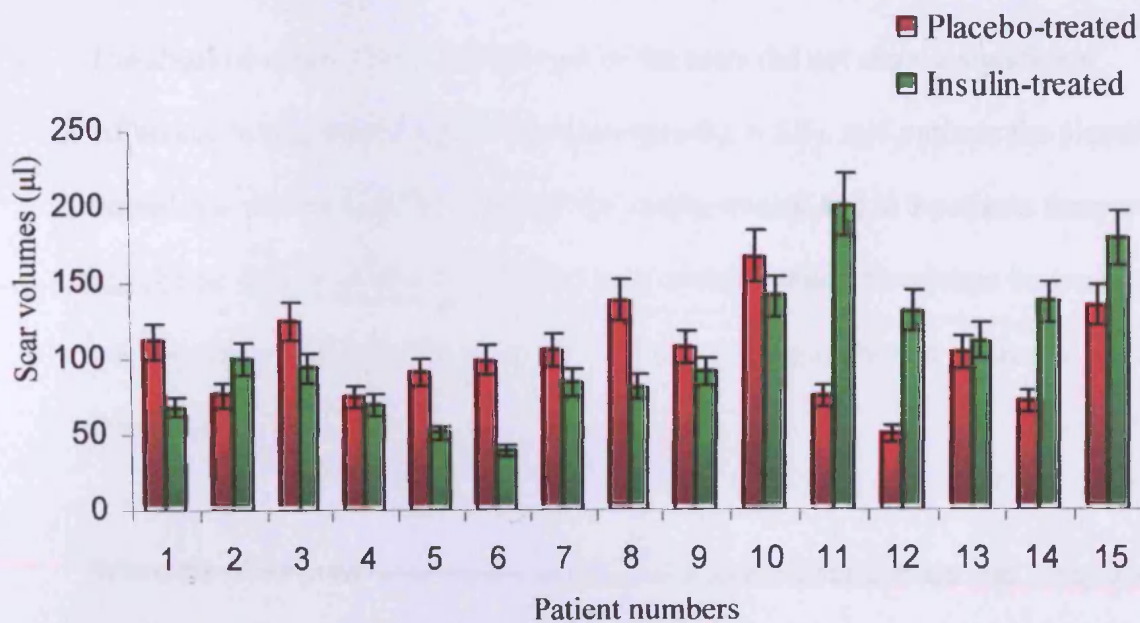


Fig. 6.5.1 The volumes (µl) of treated and untreated scars. The error bars represent the +/- 5% error inherent in this technique of volume estimation. No overall significant difference between the total volumes of placebo versus treated scars, $P=0.809$, Student paired t-test.

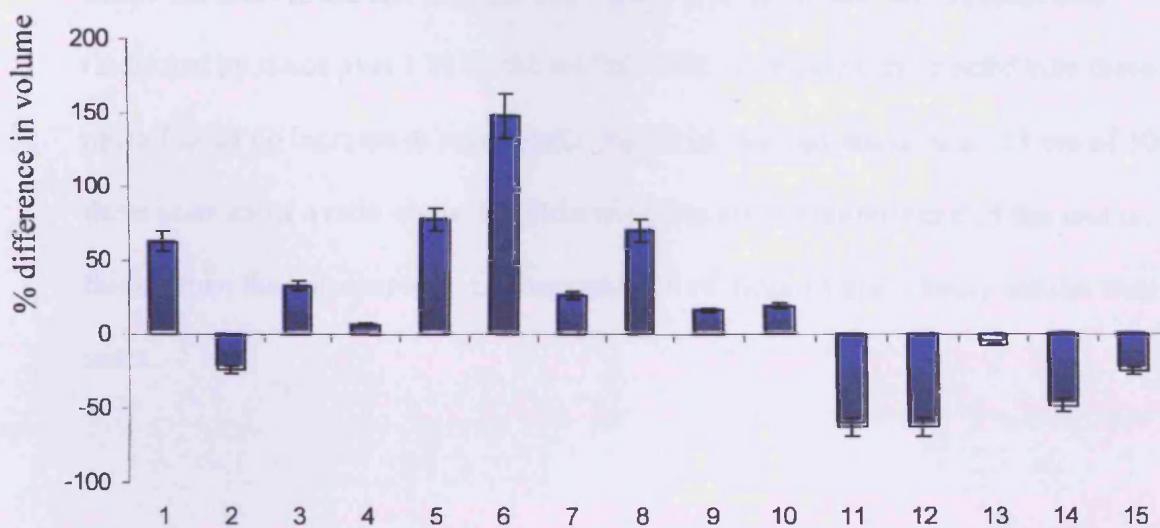


Fig. 6.5.2 The percentage difference in volume between untreated and treated scars of each patient (a positive percentage indicates the insulin-treated scar is smaller).

6.5.4 Scar prominence

The absolute values for the prominence of the scars did not show a significant difference in response to insulin treatment (see fig. 6.5.3). In 6 patients the placebo-treated scar was more prominent than the insulin-treated and in 3 patients there was no significant difference. It is notable that there is not as much correlation between scar volume and scar prominence as would be expected. Arguably scar volume is more important.

When the mean prominence of the insulin and placebo treated scars was compared to the prominence of the segments of contiguous scar that had received neither treatment nor placebo, no significant difference ($P=0.757$, paired Student t-test) was noted as demonstrated below (fig. 6.5.4). Interestingly, this graph does highlight two patients where the scars in the test area are much more prominent than the adjacent scar (indicated by ratios over 1.0) on the placebo side, whereas on the treated side there is much less or no increase in scar prominence in the test lengths of scar. 13 out of 30 of these scars show a ratio above 1.0 indicating that the lateralmost end of the scar is thicker than the adjacent scar. Unfortunately, 9 of these 13 are actually insulin treated scars.

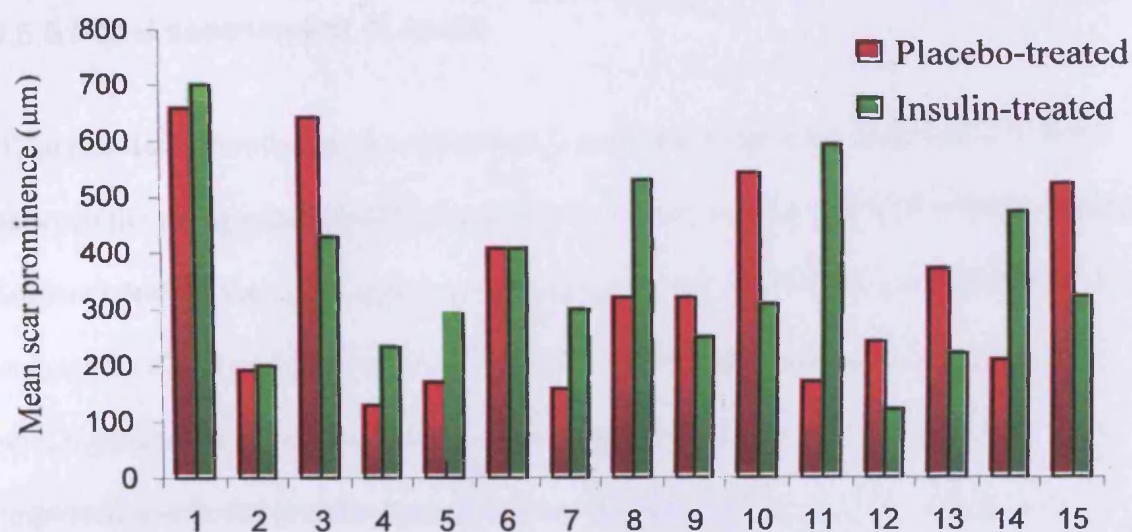


Fig. 6.5.3 The prominence (μm) of placebo- and insulin-treated scars. No significant difference seen between the two groups (paired Student t-test, $P=0.666$).

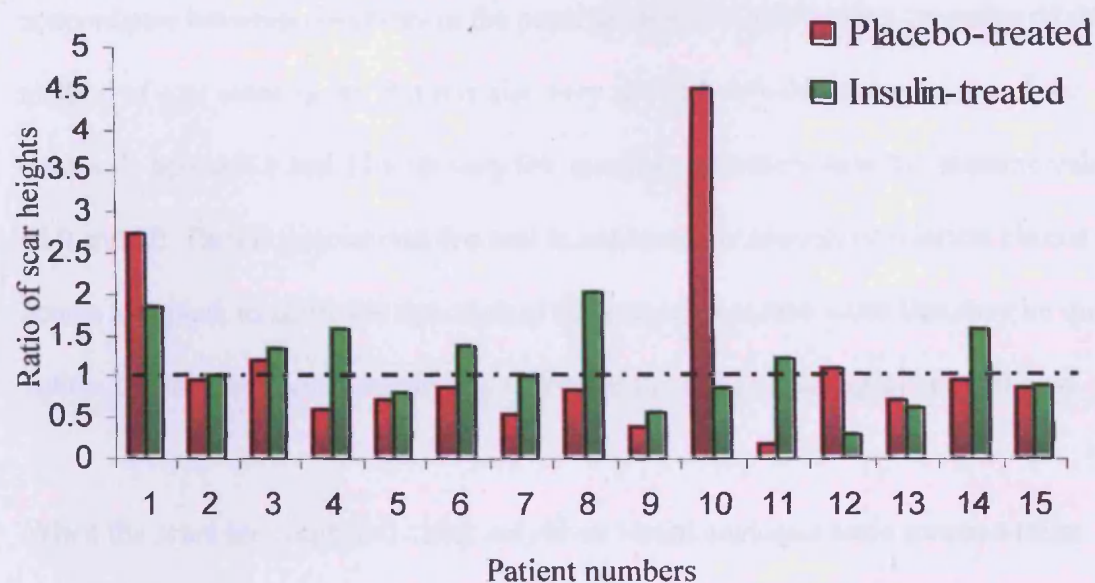


Fig. 6.5.4 The intra-scar height ratios of treated and untreated scars, no significant difference (paired Student t-test, $P=0.979$).

6.5.5 Panel assessment of scars

If the raw data from the panel assessments is analysed, there is no statistical difference between the two groups' Manchester scar scale scores (see fig. 6.5.5) ($P = 0.984$, paired Student t-test) or the visual analogue scores (see fig. 6.5.6) ($P=0.952$, paired Student T-test) or the visual analogue scores. However, the scar scale scores did show 6 patients with significantly improved scarring on the insulin-treated side, 5 patients with improved scar in the placebo-treated side and in 4 patients there was no significant difference between treatments (see fig. 6.5.5). If just the VAS scores are considered, then again six patients demonstrated significantly improved scarring on the insulin-treated wound (see fig. 6.5.6).

As is evident from the narrow error bars on the graph below, there was remarkable concordance between observers in the panel assessment, reinforcing the value of this method of scar assessment. But it is also very noticeable is that the majority of the values lie between 6 and 12 with very few reaching anywhere near the extreme values of 0 and 20. Thus it may be that this tool is not sensitive enough or restricts choice of scores too much to allow the detection of differences between scars that may be quite subtle. On the other hand, subtle improvements are not a meaningful clinical goal.

When the scars are compared using only their visual analogue scale scores a more interesting picture emerges. There is clearly much more variation between the scores for each scar and also between the scores given to the same scar by the same observers as shown by the larger error bars. The differences between patients and treated and

untreated scars in the same patients show much greater variation. Out of the 15 patients 6 have a VAS score that is lower (improved scar appear) on the insulin-treated side. Only 4 patients have better (lower) VAS scores on the placebo-treated side and the rest show no significant difference between the sides. Where there is a profound difference (over 40%) between the scars, in 3 out of 5 patients the difference is in favour of insulin.

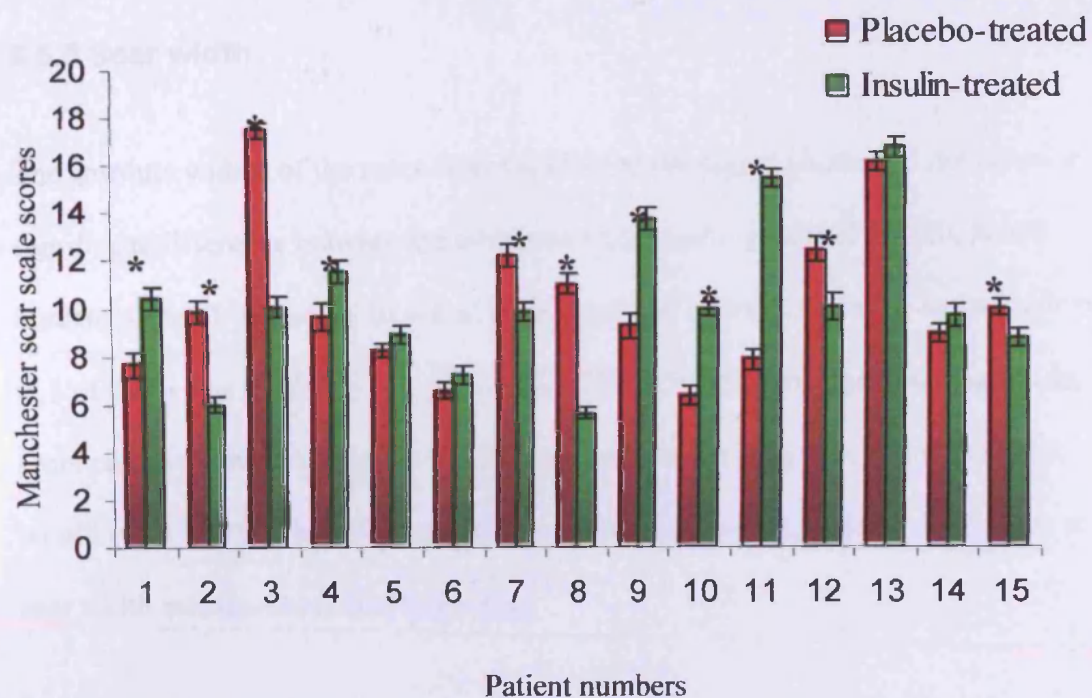


Fig. 6.5.5 The Manchester scar scale scores of scars assessed by panel assessment. the error bars represent standard error bars. There is no statistical difference between the two groups' Manchester scar scale scores ($P = 0.984$, paired Student t-test). * indicates $P < 0.05$ (paired Student t-test).

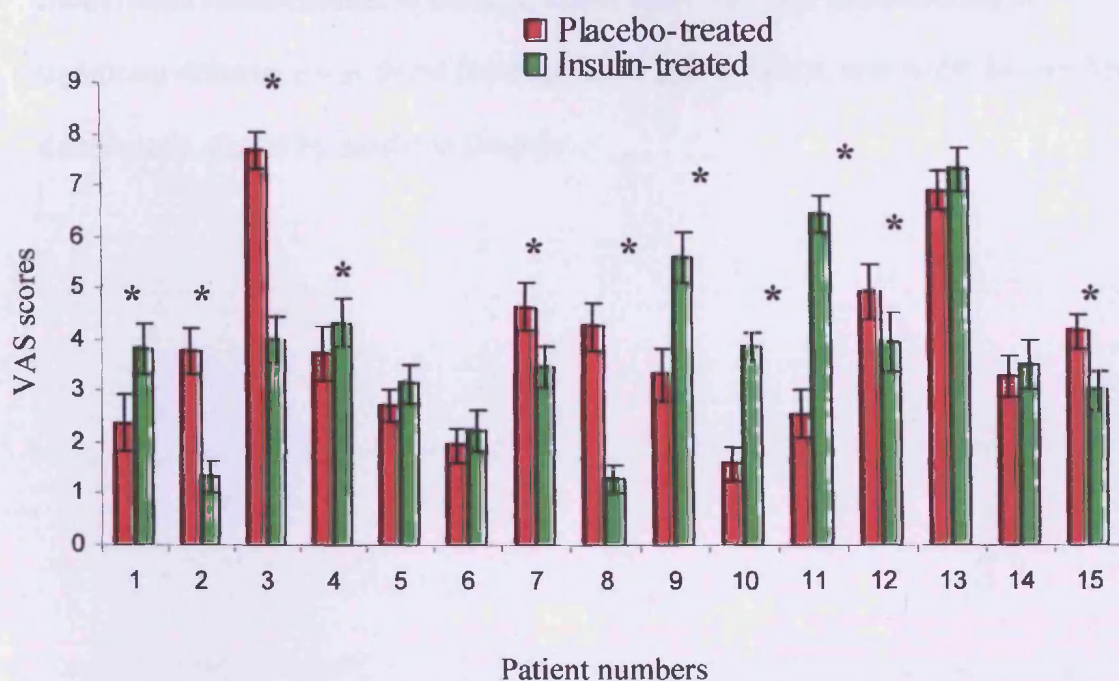


Fig. 6.5.6. The visual analogue scale scores of scars assessed by panel assessment ($P=0.952$, paired Student T-test).

6.5.6 Scar width

The absolute widths of the scars determined from the digital photos did not reveal a significant difference between the treatment and placebo groups ($P=0.760$, paired Student's t-test). Although 10 out of 15 patients had wider scars on the treated side only in 3 of these was the difference greater than 20%. Of the 5 patients which had wider scars on the placebo side, in 3 the difference was greater than 50%. Thus overall it would seem that the number of patients in which there was significant differences in scar width was the same in both groups.

When the widths of the scars in the 3cm treatment zone were compared with the widths of the same scars 1cm medial to the treatment zone and then compared with the contralateral breast (similar to the comparison made with scar prominences) no significant difference was found (see fig. 6.5.7). In conclusion, scar width has not been significantly altered by insulin in this trial.

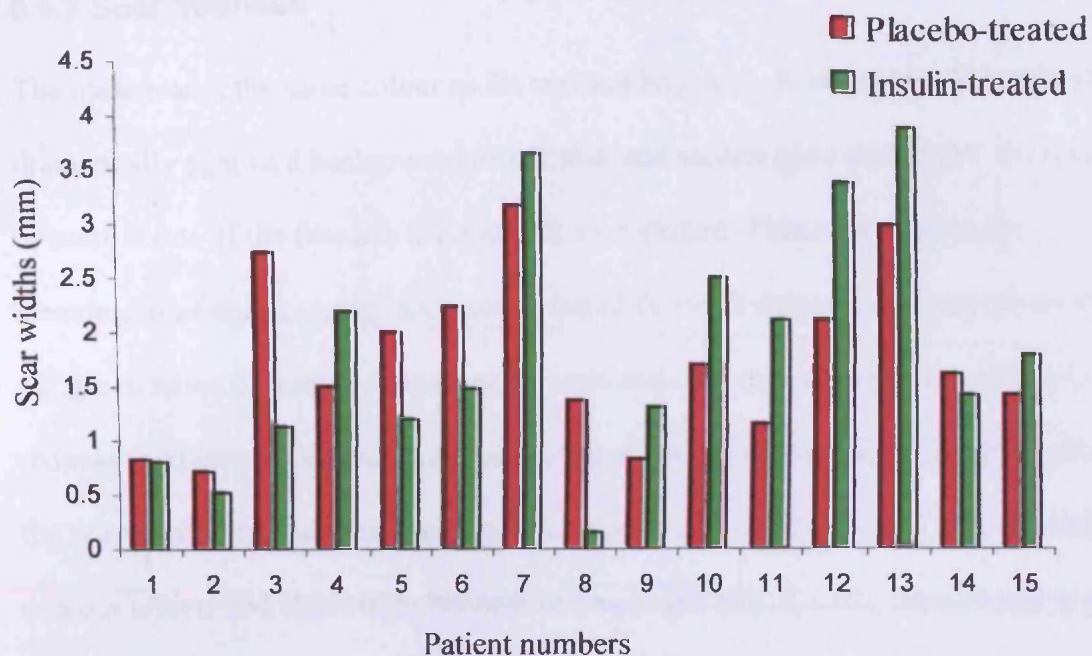


Fig. 6.5.7 The width (mm) of treated and untreated scars. No significant difference between the placebo- and insulin-treated groups (paired Student t-test, $P=0.844$).

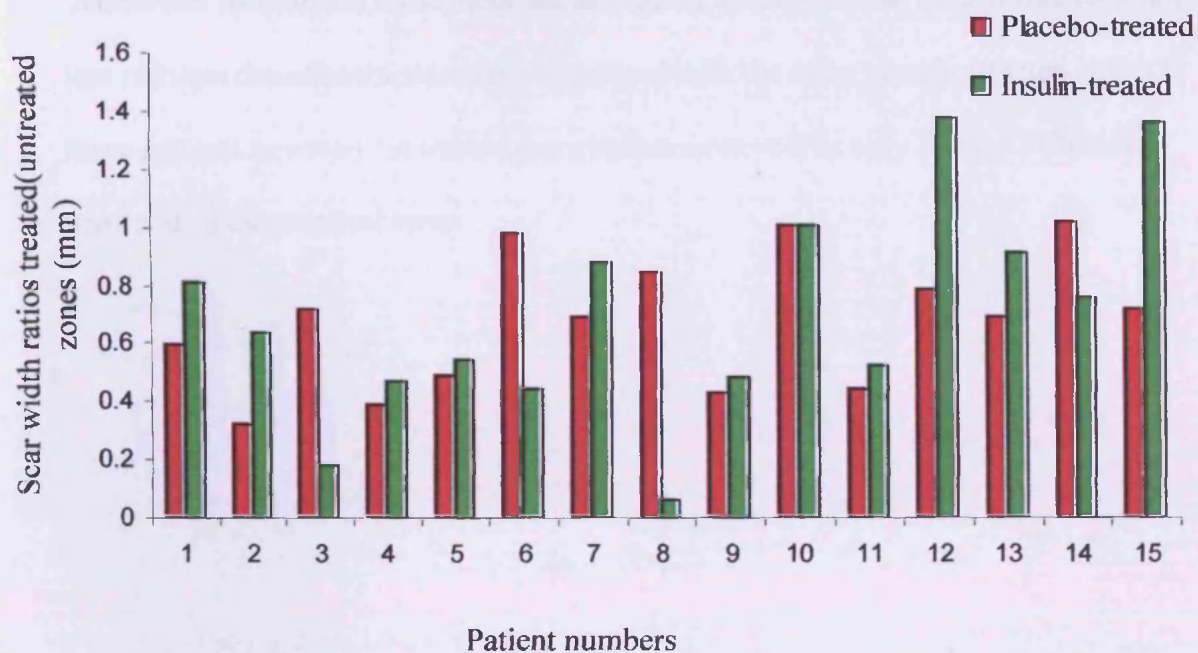


Fig. 6.5.8 Comparison of the widths of the scars in the 3cm treatment zone with the widths of the same scars 1cm medial to the treatment zone for both treated and untreated lengths of scar.

6.5.7 Scar 'redness'

The ideal scar is the same colour as the surrounding skin. A very red scar stands out dramatically against a background of fair skin and studies have shown that the redness of scars is one of the features that patients most dislike. Thus scar redness has meaningful aesthetic significance and is therefore worth measuring. Comparison of the red:green ratios of treated and untreated scars does not reveal a trend towards reduced redness/erythema in insulin treated scars (fig. 6.5.9). However, if the red:green ratios of the scars are compared with adjacent skin to control for differences in skin redness, contour effects and shadowing between left and right (fig. 6.5.10), then a trend towards reduced redness is revealed in the treated scars (placebo median 1.149, treatment 1.123; Mann Whitney rank sum test $p = 0.160$). As figure 6.5.9 indicates, only subtle differences were found throughout but in 8 out of 15 patients the insulin-treated scar is less red than the adjacent skin when compared with the other placebo-treated side. Of those patients in which the treated scar appears more red, in only 2 is the difference greater than the standard error.



Fig. 6.5.9 The red:green ratio of treated and untreated scars determined by digital image analysis. Error bars represent standard errors. There is no statistically significant difference between the two groups.

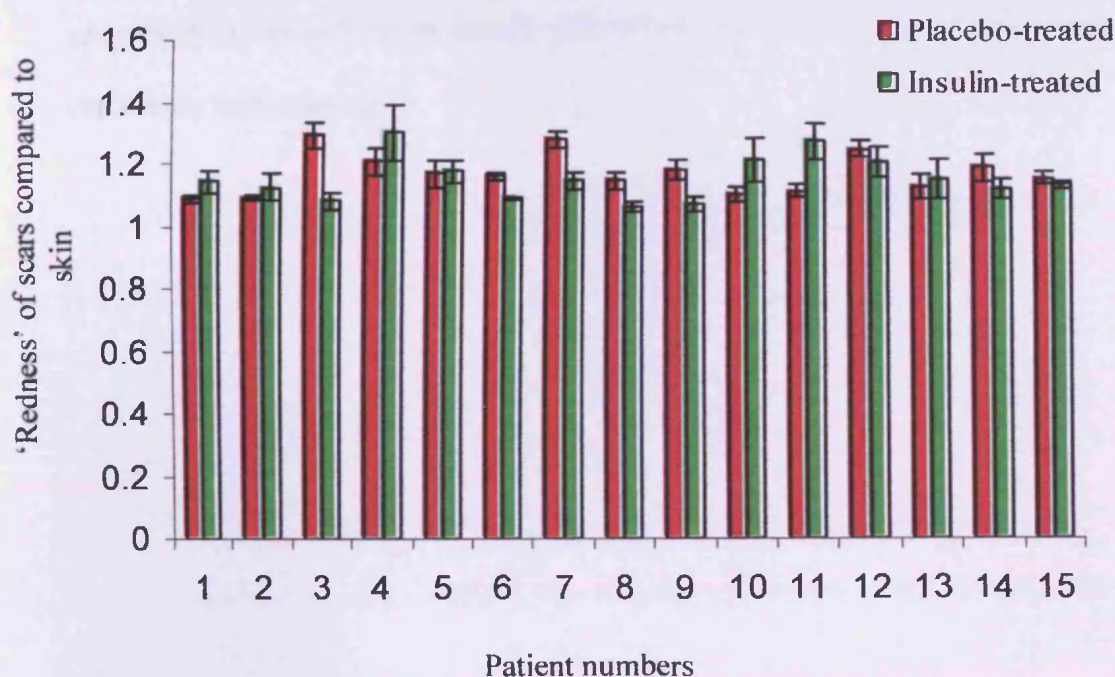


Fig. 6.5.10 The redness of scars compared to surrounding skin determined by digital image analysis. Error bars represent standard errors. There is a trend towards reduced redness in the treated scars (placebo median 1.149, treatment 1.123; Mann Whitney rank sum test $p = 0.160$) (Student paired t-test, $P = 0.528$).

6.5.8 Scar Luminosity

A shiny scar is more cosmetically unappealing than a matte scar, hence the inclusion of this characteristic in many scar scales. A shiny appearance is associated with scar activity, erythrema and hypertrophy. It is also a feature that patients are particularly concerned about, thus luminosity has also been measured.

When the luminosity of the scars was compared to adjacent skin and then compared with the contralateral breast, the luminosity of the treated scars was less although not significantly so (placebo group mean = 10.297, treatment group mean = 8.105; $p = 0.378$) (see fig. 6.5.11). Shininess is a reflection of the tautness of epithelium over a scar which in turn reflects its activity and growth which are associated with increased vascularity and redness.

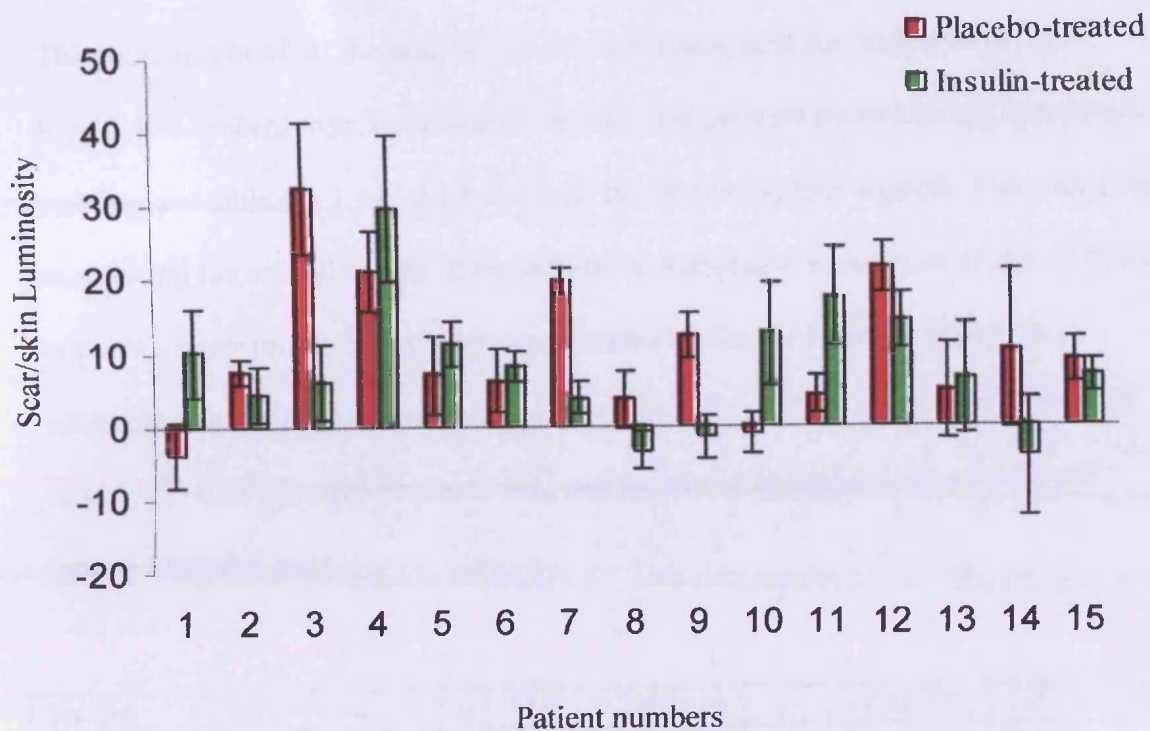


Fig. 6.5.11 The luminosity of scars (arbitrary units) determined by digital image analysis. A positive luminosity indicates the scar is 'shinier' than the adjacent skin.

6.5.9 Analysis of results with the hypertrophic scars excluded

The photographs of all the scars at the 3 month assessment are displayed in figure 6.5.12. It is evident from these images that 4 of the patients are exhibiting hypertrophic scarring and table 6.5.1 which tabulates all the results together suggests these scars may be skewing the overall results. If the results are reanalysed with the exclusion of these 4 patients a more promising impression of insulin's efficacy emerges. The panel assessment now reveals improved P numbers (in favour of insulin) for the scar scale scores – $P=0.604$ (paired Student t-test) and the visual analogue scores – $P=0.530$ (paired Student t-test).

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	I/P
Scar scale	P	I	I	P	-	-	I	I	P	P	P	I	-	-	I	6/5
VAS	P	I	I	P	-	-	I	I	P	P	P	I	-	-	I	6/5
Volume	I	P	I	I	I	I	I	I	I	I	P	P	P	P	P	9/6
Height	-	-	I	P	P	-	P	P	I	I	P	I	I	P	I	6/6
Width	I	I	I	P	I	I	P	I	P	P	P	P	P	I	P	7/8
Redness	P	P	I	P	P	I	I	I	I	P	P	-	-	-	-	5/6
Luminosity	P	I	I	P	P	P	I	I	I	P	P	I	-	I	I	8/6

Table 6.5.1. Compilation of the trial results. 'I' represents a patient in which the insulin-treated scar is 'better' for the particular property, 'P' indicates a patient in which the placebo-treated scar was better for that variable. '-' indicates a negligible difference. The final column shows the number of patients in which the insulin-treated scar was better compared to the patients in which the placebo-treated scar was better.



Patient 1 Insulin



Placebo



Patient 2 Placebo



Insulin



Patient 3 Placebo



Insulin



Patient 4 Insulin



Placebo



Patient 5 Insulin



Placebo



Patient 6 Insulin



Placebo



Patient 7 Placebo



Insulin



Patient 8 Insulin



Placebo



Patient 9 Placebo



Insulin



Patient 10 Insulin



Placebo



Patient 11 Insulin



Placebo



Patient 12 Placebo



Insulin



Patient 13 Placebo



Insulin



Patient 14 Insulin



Placebo



Patient 15 Placebo



Insulin

Fig. 6.5.12 1 to 15. Paired photographs of the placebo and insulin treated scars of all 15 patients. Note the hypertrophic appearance of the scars in patients 3, 9, 11 and 13.

6.6. Discussion and conclusion

The comparison of the two formulations Insulatard® and insulin glargine generated two important findings: firstly it reproduces the finding of insulin's myofibroblast inhibiting actions and secondly it demonstrates the efficacy of two commercially available formulations licensed for use in humans that are immediately available and suitable for use in a clinical trial.

However, just as differences in the efficacies of shorter acting insulins and Insulatard® were seen (Mackie 2004), there are also differences in the efficacies of Insulatard® and insulin glargine. The difference in concentration at which the two formulations are most effective may be due to their differing pharmacokinetic profiles. As Insulatard® reaches a peak activity/availability in the first 12 hours in contrast to the constant activity/availability level over 24 hours that insulin glargine exhibits, for a given dose, Insulatard® must reach higher peak activity levels. This might explain why Insulatard® is effective at a lower dose than insulin glargine. However, the pharmacokinetic profiles of these formulations may differ in the in vitro and in vivo environments and this would be demonstrated by inclusion of both drugs in a clinical trial. However, there may also be differences between the stabilising solutions. For example, the commercial preparations contain varying levels of insulin stabilisers such as zinc, which is known to improve wound healing (Udupa et al. 1971).

Although overall there does not appear to be any great dissimilarity between their effects, Insulatard® is effective at the lowest concentration. Therefore, following the principle that a drug should be used at the minimum effective concentration so as to reduce the risk of side effects, Insulatard® would be the preferred choice for use in a clinical trial.

Although the structure of recombinant human insulin such as insulin glargine that were used in this chapter differ slightly from the structure of bovine insulin used in the rest of the thesis and in preliminary studies by Linge et al 2004 and thus may have different biological effects and perhaps more efficacy in the in vitro experiments all three formulations were found to be very effective at inhibiting myofibroblast differentiation. Both Insulatard® and glargine showed greater efficacy with increasing concentrations. Insulin never displayed absolute efficacy that is, reducing myofibroblast numbers to zero however, even in excess of physiological levels. It was only in freshly seeded cultures examined at T0 that myofibroblasts were practically absent. Furthermore, all of the formulations of insulin, including bovine insulin, rarely succeeded in bringing myofibroblast numbers below those observed in the control cultures in NGM. This is interesting because it implies that some myofibroblasts are refractive to this action of insulin due to differences in either sensitivity to insulin or in the plasticity of the differentiated state. It is further possible that the persistent myofibroblasts represent another cell type, such as pericytes, in with the fibroblast cultures that also express α -SMA irrespective of the presence of insulin.

It is notable that the maximum efficacy of the human formulations of insulin occurred at supraphysiological concentrations (physiological levels are 6 -26 μ IU/ml (Entwistle 1990), maximum effect in experiment at 0.5-1.0IU/ml). These doses may achieve saturation of the insulin receptor with fast-release formulations of insulin but this is less likely with the slow-release medications. Perhaps receptors other than the insulin receptor are involved in its action.

Overall the results show a trend towards reduced luminosity (20% reduction) (see fig. 6.5.11) and redness (see fig. 6.5.9) in the treated scars. There is also a weaker trend towards smaller volumes in the insulin-treated scars (9 out of 15 patients) (see fig. 6.5.1) and 6 out of the 15 patients displayed significantly better scarring on the insulin treated sides according to the panel's assessment of Manchester scar scale scores and VAS scores (see figs. 6.5.5 and 6). The other methods of assessment of the scars, namely measurement of scar prominence and width did not show any significant difference between the two groups. The general conclusion is that there is a tentative suggestion that insulin may marginally affect some, but not all aspects of scarring although further trials are clearly required before a definitive conclusion can be reached.

Unfortunately, it can be concluded with much greater confidence that Insulatard[®] doesn't prevent the formation of scar tissue and allow scarless healing. However, this is in keeping with the in vitro work that indicated that although insulin reduces myofibroblast numbers in fibroblast cultures, it doesn't reduce their numbers to zero.

Furthermore, Insulatard® doesn't appear to make normal scars 'better' in the short term (3 months), although the study also reiterates the finding that comparing normal scars is very difficult. Reanalysing the results excluding the 4 patients that had hypertrophic scarring strengthens the trends in favour of insulin although the results remain far from significant. This therefore suggests that insulin has little effect on pathological scars but may improve normal scars. This suggestion may influence future laboratory investigations in terms of the type of cell used (normal scar rather than hypertrophic) and continuing the research into insulin's mechanism of action. In addition this implies that future trials should specifically use normal scarring scenarios (by far the majority of scarring scenarios) such as excisions of benign skin lesions. This knowledge will also be of great use in estimating sample sizes for future trials and in choosing the best scar assessment techniques, that is those that are sensitive enough to distinguish between normal scars.

The scar characteristics that insulin may have affected, that is luminosity, redness and volume are perhaps more reflective of scar activity/inflammation than the other features such as width and prominence. This would be consistent with insulin's possible counter-TGF- β 1 activity.

These conclusions are being drawn for Insulatard® and insulin generally but could these outcomes be dose specific? Could Insulatard® actually prove much more effective at higher doses? There's good evidence from the in vitro work and previous work (Mackie

2004) that insulin's and Insulatard's® effects are dose dependent with greater efficacies at higher doses, particularly 0.5 to 1.0 IU/ml. clearly in this trial such concentrations would not have been reached in the wounds where arguably even higher doses would be needed anyway to compensate for diffusion and perfusion. Therefore, without further trials testing different doses, the conclusions from this trial must remain specific to Insulatard® at this specific dose.

Although demonstrating Insulatard's® safety was not an issue in this trial as it has already been proven, the study did demonstrate that Insulatard® does not hinder wound closure nor worsen scarring. This evidence will be helpful in the planning of further trials especially in terms of gaining the approval of local and national ethics committees.

The summary conclusion is that Insulatard is safe for further trials of this application but that its efficacy in scar inhibition has not been proven.

6.6.2 Sources of error

The factor that has perhaps contributed most to an indefinite result has been the overall quality of scars whether treated or untreated. Evidence of efficacy is far more detectable if the untreated wounds develop poor scars. An improvement in scars that were already predestined to be excellent would require more sensitive measures than those used. Furthermore, if insulin inhibits hypertrophic scarring in addition to normal

scarring, then the low incidence of hypertrophic scars overall makes it difficult to demonstrate such efficacy in a study of this scale.

Related to this is the sample size. The estimation of sample size was based on the assumption that hypertrophic scarring would be more common than it actually was and that insulin's efficacy would be greatest in relation to hypertrophic scars rather than normal scars. The assumption was also made that insulin would have a large effect. However, a large effect is only demonstrable when there are a reasonable number of hypertrophic scars in the placebo group. Thus the required sample size was inevitably underestimated. In practise though, there were not enough patients to recruit at the hospital in the time involved to generate a larger sample size.

The techniques for measuring individual parameters also carry with them some degree of error. The estimation of scar volumes gets increasingly difficult and less accurate as the scars become smaller. However, the method is straightforward and reasonably accurate in the measurement of the larger, hypertrophic scars, so if there was a significant effect in the incidence of hypertrophic scars then this method would seem to be sensitive enough to detect it. Experimental digital imaging and 3-D reconstruction techniques would be the more accurate ideals for the future.

The prominence of the scars was determined from two measurements taken from each mould which may inaccurately reflect the height of the full length of scar. The technique is complicated and requires some degree of subjectivity in identifying the

real peaks and troughs of the moulds. Ultrasound would perhaps be more effective if user expertise was sufficient. Measurements of scar prominence are always going to be challenging because the dimensions involved are so small and the scar cannot be detached from the skin.

Measurement of scar widths was the most accurate and reproducible technique. The one source of error is in the judgement of the margins of the scar. In the red hypertrophic scar this is straight forward but in a fine pale scar this can be difficult. However, the technique is surely sensitive enough to distinguish between a 'good' and a 'bad' scar.

Analysis of scar redness and luminosity although straight forward is less accurate for the thinner scars and is of less importance than the panel assessment findings and estimations of scar volume. It is however, very reproducible especially in the larger scars and brings additional parameters that can be objectively measured.

The panel assessments of the scars, although seemingly the most subjective of methods, are probably the most useful results. The scores show remarkable interobserver concordance, the scale is sensitive enough to easily distinguish between good and poor scars, accuracy is easily increased with additional assessors and multiple parameters are assessed simultaneously. However, possible sources of error from this method are changes in assessors' judgements that occur during the course of the assessments as they become more experienced and confident. In addition despite the scoring system for individual parameters different assessors may use their own criteria (unintentionally)

for assessing the scars or may have different opinions regarding which properties make a scar good or poor. The scar parameters that are individually assessed such as colour, distortion, shiny/matt allow as little choice as 1 or 2 meaning that these sections of the scale do not distinguish very much at all between different scars and this really is the main failing of the Manchester scar scale. Another possible source of error is the inclusion of laypeople and scientists into the panel with surgeons, but in fact this does not appear to have led to discordant results. The finding that a variety of people are making the same judgements about the scars probably gives greater credibility to the conclusions.

6.6.3 Repetition of the trial

If the study were to be repeated a larger sample size would definitely be required, probably between 50 and 100 patients. Rather than using the lateral ends of the breast scars, the medial ends would be better. This is because we've shown insulin doesn't make the scars disappear entirely so visible asymmetry is not a problem and also because the incidence of hypertrophic scarring is higher medially.

Alternative procedures could also be considered or several different procedures included in the trial. For double incision procedures in addition to bilateral breast reduction surgery, bilateral augmentation mammoplasties, bilateral carpal tunnel decompressions and bilateral otoplasties could be included. Procedures involving large incisions that cross midline could be used and the ends treated with insulin or placebo.

This would apply to abdominoplasties, apronectomies, breast reconstruction free flaps and facelifts.

Future trials could be directed more specifically towards either normal scarring as stated above, or hypertrophic scarring. In the latter case procedures such as revisions of pathological scars, incisions in anatomical sites prone to pathological scarring (for example, midline sternotomy wounds), burn scar revisions, scars crossing joints and patients with positive histories would be appropriate. Ultimately, pending advances in insulin drug delivery, acute burn excisions could be included or even conservatively managed burns. This could even extend to trials of systemic insulin in patients needing intravenous resuscitation post burn injury although this would be ethically more challenging because of the inherent risk of hypoglycaemia associated with systemic insulin treatment.

A higher dose of Insulatard[®] or a variety of doses and formulations would be essential. It may be that the dose used in this trial simply wasn't high enough. Human dermis is rich with capillaries and much thicker than murine; therefore the Insulatard[®] may well be being absorbed into the general circulation too quickly for effect and need higher doses per unit area of skin. Repeat injections or longer acting insulins might be required despite the conclusions from the murine model. Furthermore, the in vitro experiments show that insulin has varying antifibrotic across different cell lines (patients) (REF) so it may be that the optimum dose varies significantly from patient to patient.

The possible problem of insulin dispersing throughout the tissues and affecting a placebo-treated wound in close proximity is considered unlikely because where insulin treatment had markedly improved the scar there was a distinct demarcation of where it had been applied.

In the assessment of scars digital image analysis would still be helpful but panel assessments using scar scales would probably be the most effective tool. They show superb interobserver concordance, they're easy and cheap to carry out and they give an overall impression of scar appearance that is actually the most important assessment anyway. The ideal, gold-standard assessment would be excision and histological examination but this method is more suitable for testing the final product following phase II, III and IV trials.

There is increasing evidence that the effects of an antiscarring agent administered at the time of wounding do not necessarily become apparent until after 3 months. Therefore, follow-up in future trials should ideally be longer than 3 months and indeed should continue until scar maturity at 18 months. Arguably longer follow-up still could be desirable, especially in the context of burns or other hypertrophic scars as scarring is a dynamic life long process it could be that insulin can delay the onset of hypertrophic scarring but not necessarily its final outcome.

Once the most effective dose and formulation of insulin or a derivative has been established by clinical trials then the further trials could be established to test different

methods of administration. For example, gels, creams, patches, sprays and drug iontophoresis could be tested in the context of different types of burns, ulcers and complex wounds. The trials could also be extended to include atrophic and keloid scars and also to examine the efficacy of insulin in combination with other antiscarring therapies.

6.6.3 Conclusion

1. The increased myofibroblast differentiation in growth factor deactivated media is inhibited by bovine insulin and the human formulations Insulatard® and insulin glargine with greatest efficacy seen at high and supraphysiological concentrations.
2. The long-acting human formulation of insulin, Insulatard®, significantly inhibits myofibroblast differentiation at a lower concentration (0.5IU) than insulin glargine (very long-acting) or shorter-acting insulins and is therefore the most suitable formulation for a clinical trial (fig. 6.2.1).
3. Insulatard® is a safe drug to use for the prevention of poor scarring and demonstrates no adverse effect on wound closure or scarring.
4. Three new objective methods of assessing scars (multiphoton microscopy and silicone moulds, digital image analysis) have been demonstrated and show reliability.
5. Insulatard® appears to be potentially efficacious at affecting normal and not hypertrophic scars.
6. Scars treated with Insulatard® show trends towards having smaller volumes (figs. 6.5.1 and 2) and show trends towards being less red (figs. 6.5.9 and 10) and less shiny (fig. 6.5.11).
7. A much larger scale trial is needed to clarify whether the observed trends are significant.

Chapter 7

Discussion and conclusion

7.1 Discussion

The aim of this thesis was to expand the work performed by Linge et al in 2004 in order to determine the mechanism of action of insulin as an anti-scarring agent and demonstrate its efficacy in vivo in humans in a clinical trial. This thesis has demonstrated the myofibroblast inhibitory effects of insulin and subsequently refuted many possible mechanisms of insulin's actions. However, the results presented here have also pinpointed a potential mechanism of action that requires further study. The pilot clinical trial suggests that insulin may have some efficacy in vivo as an antiscarring agent and its safety has been confirmed and new methods of scar assessment described.

Although insulin has never been tested as anti-scarring agent before, continuous topical or systemic application of insulin has been shown to significantly accelerate wound healing (in non-diabetic patients) (Greenway et al. 1999) (Pierre Barrow et al 1998) (Weringer et al. 1982). Topical insulin is also known to significantly improve wound-healing in diabetic subjects whilst systemic insulin reverses the impaired wound healing associated with diabetes mellitus (Anderson and Oxland 1987) (Goodson and Hunt 1978). Such systemic treatment is effective if started before injury or during the first week after injury. Greenway et al. (1999) found that standard wounds treated with insulin healed 2.4 ± 0.8 days faster than control wounds and finally, Pierre et al 1998 demonstrated that systemic insulin can accelerate the healing of large burns.

The effects of subcutaneous supraphysiological doses of insulin are well known from the experiences of diabetics who daily inject very high concentrations of insulin frequently into the same body site. These sites do not show impaired wound healing at the sites of insulin injection although they do over time show lipoatrophy. Conversely there is no fibroblast hyperplasia or tumorigenic response although rarely benign lipohypertrophy is seen at the sites of injection.

There have been myriad developments in surgical techniques, preventative measures and treatments that attempt to tackle pathological and normal cutaneous scarring (see chapter 1) and although considerable progress has been made in the understanding and treatment of scarring, there is still no effective preventive anti-scarring agent. There are therapies such as silicone gel and intralesional steroid injections that can ameliorate the excesses of scarring but they are not curative. There is therefore a need for an agent that can be administered at the time of injury or operation that can inhibit subsequent scar formation. Ideally, it should also be cheap, safe, non-allergenic, easily administered and not require repeat dosing. Insulin, or a drug derived from it, could potentially meet all of these requirements.

7.1.2 The influence of the test media

The results chapters began with a discussion of the different culture media that were going to be used in this thesis (see section 3.1). Central to this discussion are two points. Firstly, that it was in growth factor deactivated media that myofibroblasts were observed in increased numbers (Linge et al., 2004) and secondly that for the type of

prolonged culture experiments that were going to play a part during the research the ideal growth media should be those that permitted only a low proliferation rate. These thoughts became more relevant with the general observation during some of the initial experiments that the health of cell cultures appeared to be impaired in DTTM particularly in respect to cell proliferation while insulin seemed to lessen this effect. Although dithroeditol is known to be toxic to cells, DTTM was used in this research because of the low rates of cell proliferation observed in it (Linge et al., 2004). This is a desirable property because it allows prolonged experiments that are not overwhelmed by cell proliferation and because it reduces the risk of changes in the proportion of myofibroblasts being masked by greater changes in fibroblast proliferation. However, the potential toxicity of DTTM led to the use of other test media. But as figure 3.3.2 demonstrates there is no significant inhibition of fibroblast proliferation in DTTM compared to DFCS thus permitting comparisons between the two. Additionally, although proliferation is undoubtedly increased in NGM compared with these media, this increase is not significant before day 14. Importantly, addition of insulin to these media was associated with only a minimal increase in proliferation indicating that its effect is not simply a misinterpretation of differences in proliferation. In contrast addition of insulin to NGM did lead to a significant increase in proliferation (fig. 3.3.2).

7.1.3 Insulin inhibits the induction of myofibroblast differentiation

Reassuringly, section 3.2 demonstrated the reproducibility of the initial experiments performed by Linge et al 2004 that recognised that myofibroblast numbers are

increased in growth factor deactivated media (approximately 25% of cell numbers by day 14) compared to normal growth medium (5%) (see fig. 3.2.2). Again reproducing the original work of Linge et al. (2004) insulin was shown to abrogate this phenomenon. Interestingly, the experiments reported in this thesis using normal scar fibroblasts demonstrate a higher proportion of myofibroblasts (25% at day 14 in DTTM) compared to the number reached (10%) when dermal fibroblasts were used in an equivalent experiment performed by Mackie (2004). This may represent intrinsic differences in responsiveness between the two cell types. Importantly, section 3.4 demonstrated that neither short (4 days – fig. 3.4.1) nor prolonged (14 days – fig. 3.4.2) insulin treatment would reduce the numbers of myofibroblasts in fibroblast cultures if exogenous active TGF- β was simultaneously or subsequently added. That is, while active TGF- β is present in the medium, insulin is unable to counteract TGF- β 's pro-myofibroblast differentiating effects regardless of the prior duration of insulin treatment. However, this finding is specific to active TGF- β , as autocrine TGF- β is secreted in an inactive form. These results suggest that insulin does not appear to affect either the differentiation process per se nor TGF- β 's induction of differentiation. These results are supported by another series of experiments that were being performed at the same time where the effect of insulin on a number of different components required (prerequisites) for successful myofibroblast differentiation were being examined (see the following section).

7.1.4 The potential mechanisms of action of insulin that are independent of TGF- β

The experiments that examined potential mechanisms of insulin's action that do not directly involve TGF- β were undertaken synchronously with the other lines of research. However, during the course of the research evidence mounted that supported the hypothesis that autocrine TGF- β was implicated in insulin's mechanism of action and therefore efforts were concentrated on this direction rather than fully completing these other investigations.

The role of TGF- β is at the forefront of scarring research and therefore inevitably has been perceived throughout this research as a strong contender for having a prime involvement in insulin's mechanism of action. However, there are other potential mechanisms that are independent of TGF- β and that needed to be addressed too. These mechanisms can be grouped together in simple terms as those that directly inhibit the fibroblast-myofibroblast differentiation process by reducing the expression or actions of proteins involved in that process. EDA-fibronectin is the most important example of such a protein, the presence of which is a prerequisite for the induction of differentiation (Grotendorst et al. 2004). However, insulin treatment did not inhibit the expression of this protein in terms of either immunostaining or Western blotting, in fact there was even a trend towards increased EDA-fibronectin expression in the insulin treated fibroblasts (see figs. 3.5.1-3).

The expression of stress fibres precedes the appearance expression of α -SMA. Figure 3.6.1 shows that insulin does not inhibit the expression of stress fibres. This implies that

insulin's action specifically inhibits α -SMA expression rather than the expression of any of the other stress fibres which in turn implies a very specific mechanism of action. Related to the expression of stress fibres is the expression of FAK kinase and its activated form phosphorylated FAK kinase which are involved the mechanosensory roles of the focal adhesion complex (Endlich et al. 2007). Figure 3.7.2 suggested a possible trend of less FAK kinase staining in insulin-treated fibroblast cultures. If repetitions of this experiment found this to be a significant trend (and supported by Western blotting analysis) then this would indicate a possible mechanism of action for insulin although disruption of the focal adhesion complex assembly might lead to myofibroblasts that fail to contract but do still produce volumes of extracellular matrix. However, as figure 3.7.1 shows, insulin does not appear to affect the number of cells staining positively for phosphorylated FAKinase.

Unfortunately Western blotting analysis of these proteins did not yield persuasive results but immunostaining did show that there was no significant affect from insulin treatment. Although immunostaining is an inherently unsatisfactory way of judging the levels of expression of a protein, a lack of obvious effect of insulin was consistently seen across a range of proteins which suggests that the possible mechanisms of action that these proteins are involved in are unlikely to be involved in the very significant reduction in α -SMA immunostaining that is seen with insulin treatment. If insulin were to inhibit the production of FAK kinase or EDA-fibronectin for example, this could prevent the differentiation of any myofibroblasts at all which it could be expected to have adverse consequences for wound healing and strength – features that are not

observed in either the murine (Mackie, 2004) or human model (see chapter 6).

Conversely, insulin's action does not appear to simply to interact solely with α -SMA as TGF- β will induce α -SMA positive myofibroblasts even in cultures exposed to prolonged insulin treatment (see fig. 3.4.2).

Other potential mechanisms of action were also explored including Thy-1, thrombospondin, integrin and adipocyte differentiation. Thy-1 status has been demonstrated in non-dermal fibroblasts to determine the potential to become myofibroblasts if Thy-1 positive (or if Thy-1 negative preferentially differentiate into lipofibroblasts) without actually being involved in the differentiations steps (Koumas et al. 2003). All cells examined appeared to express Thy-1, and this did not seem to be influenced by insulin treatment (see fig. 3.8.1). This finding is corroborated later in this thesis by the demonstration that insulin does not induce fibroblasts to differentiate preferentially into an adipogenic rather than myofibroblastic phenotype (see fig. 3.9.1). Koumas et al. found that only Thy-1⁻ human myometrial fibroblasts differentiated into lipofibroblasts whereas only Thy-1⁺ fibroblasts were capable of differentiating into myofibroblasts (Koumas et al. 2003). Although human fibroblasts have not been demonstrated to differentiate into adipocytes, mouse fibroblasts have been shown to do so in response to troglitazone (an anti-diabetic drug that decreases insulin resistance) (Liu et al. 2004). It is fortunate that this potential mechanism of action of insulin was not supported as differentiation of wound fibroblasts into adipocytes would likely have had adverse implications for the tensile strength of healed wounds.

7.1.5 Insulin does not affect TGF- β expression

The next stage of the research was to see whether insulin affected differentiation of myofibroblasts indirectly, that is via action on autocrine TGF- β . The TGF- β isoforms are the subject of most of the current attempts to produce an anti-scarring therapy (Shah, Foreman and Ferguson 1995) (Shah, Revis et al 1999). Notwithstanding the concerns about the manipulation of TGF- β isoforms particularly because of the potential deleterious effect on other aspects of wound healing, there remains a need for further investigation of other potential anti-scarring therapies whose mechanisms of action do not completely block TGF- β throughout wound healing. Irrespective of these concerns TGF- β is the crucial growth factor that orchestrates scarring and therefore remains a potential mechanism for the action of insulin.

The investigation of this began with RT-PCR analysis of fibroblasts cultured in growth factor deactivated media with and without insulin using TGF- β 1 primers (see section 4.2). Insulin did not affect the mRNA expression of TGF- β 1 in these cultures (see figs. 4.2.1-2) or even in cultures of 0.4% FCS that were treated for 3 days with exogenous TGF- β 1 (fig. 4.2.2). This lack of effect of insulin on TGF- β 1 gene transcription was also seen for levels of TGF- β 1 protein detected via ELISA (see fig. 4.4.1). In addition later experiments in chapter 5 also demonstrated that insulin did not significantly affect the protein expression of the small or large latent TGF- β complexes either or indeed the small amounts of active TGF- β detected (albeit these levels were highly variable (see figs. 5.3.4-8). Overall these results strongly suggest that insulin does not affect TGF- β 1 expression.

The importance of the other TGF- β isoforms TGF- β 2 and 3 has been highlighted by others (Ferguson et al., 2004). However, RT-PCR analysis of insulin's effects on the expression of these two isoforms showed that they were present in extremely low levels in these cultures and this was confirmed by real time RT-PCR (see table 4.3.1 and fig. 4.3.1). Therefore it is unlikely that alteration of the TGF- β isoform mRNA expression is insulin's mechanism of action. However, the protein products of growth factor genes have very high bioactivity so it is possible that the TGF- β 2 and 3 isoforms do still have some role to play with perhaps greater changes occurring at the level of protein translation, cellular distribution or changes in receptor expression, distribution or interactions. It is certainly true that therapies that exploit the influence of the TGF- β isoform ratio will potentially lead the way in the next generation of anticancer therapies (Ferguson et al. 2004). If insulin does have a mechanism that is completely independent of the balance of TGF- β isoforms, the final drug may be suitable for a combination therapy approach which has proved successful in other fields of medicine such as cancer chemotherapy.

Although insulin does not appear to affect TGF- β expression, section 4.5 hints that insulin may well affect TGF- β 's action. Figure 4.5.1 showed that insulin could inhibit further myofibroblast differentiation *after* exogenous TGF- β was removed from the medium although this effect took approximately 7 days to become noticeable. These experiments taken together suggest that insulin cannot inhibit the induction of myofibroblast production by activated TGF- β but can inhibit its subsequent inducing

effects after it is no longer present. These findings have implications for insulin's mechanism of action, that is insulin may act by inhibiting the downstream effects of activated TGF- β or perhaps by altering its expression or interaction with its receptors.

This increase can be reduced by the addition of insulin though, despite 3 days pre-treatment with exogenous TGF- β 1 (see fig. 4.5.1). However, this effect of insulin occurred after TGF- β 's removal from the media, insulin did not appear to inhibit the increase in myofibroblast numbers that occurred when TGF- β 1 was present or in the early period after its removal (up to day 7). Furthermore, insulin could not inhibit the myofibroblast-inducing effects of exogenous TGF- β 1 whilst it was present but could inhibit the subsequent increase in myofibroblast numbers that occurs after (fig. 4.5.1). The important point here is that the second increase in myofibroblast numbers that occurs *after* the removal of TGF- β is due to the action of activated autocrine TGF- β . It is this activated autocrine TGF- β that insulin appears to be inhibiting. There is little difference between paracrine and autocrine TGF- β apart from the fact that the paracrine form reaches the fibroblast in an activated state whereas the fibroblast must activate the autocrine form. This therefore suggests that insulin's mechanism of action is via inhibition of the activation of autocrine TGF- β 1.

Repetition of the experiment detailed in 4.5 with fetal dermal (rather than adult normal scar) fibroblasts (see section 4.6) with either TGF- β 1 or TGF- β 1 and insulin added for the first three days only revealed that whilst insulin did not appear to reduce myofibroblast numbers (see fig. 4.6.1), TGF- β 1 did not by itself produce the same

persistently raised myofibroblast numbers beyond day 7 that are seen in that are seen adult fibroblasts (see fig. 4.5.1). In fact, the insulin treated adult fibroblasts have the same pattern of myofibroblast differentiation over time as the TGF- β 1 treated (+/- insulin) fetal fibroblasts. Although the experiment was limited to only one fetal cell strain, this short-lived myofibroblast differentiation response to TGF- β 1 has been reported before (Rolfe et al. 2007a). Nevertheless this experiment needs to be repeated with greater numbers before any firm conclusions can be drawn as to insulin's efficacy on fetal cells. These observations prompt speculation that insulin exerts its effects by stimulating a 'fetal' pattern of scar formation in adult skin.

7.1.6 Insulin affects the activation of autocrine TGF- β 1

Earlier findings in the research hinted that insulin's mechanism of action might involve the activation of autocrine TGF- β 1. Section 4.5 demonstrated that a transient treatment with TGF- β led to sustained myofibroblast numbers over a long period of time and insulin treatment reduced myofibroblast numbers at later time-points but no decrease was seen in the initial levels reached. Therefore components known to be involved in the activation of TGF- β were examined. Both the integrins (specifically α V β 5) and thrombospondin activate TGF- β although their exact roles in relation to the differentiation of myofibroblasts are still being clarified. Immunostaining of thrombospondin was unfortunately unsuccessful. Immunostaining of α V β 5 integrin was more successful and indicated a possible trend towards its increased expression in insulin-treated fibroblasts (see figs. 5.5.1 and 2) but unfortunately Western blotting

analysis did not yield results that could support this finding one way or the other.

Clearly much more investigation of the integrins is required including those integrins that may activate TGF- β such as $\alpha V\beta 1$, $\alpha V\beta 6$ and $\alpha V\beta 8$ but also $\alpha V\beta 5$. MT1-MMP, the metalloprotease involved in the $\alpha V\beta 8$ mediated activation of TGF- $\beta 1$ LAP and also proteolytic activation of TGF- $\beta 1$ LAP (Mu et al., 2002), also deserves more investigation.

In addition to the means of activation described above, TGF- β is also activated by the IGF-II receptor. The role of this receptor was examined with the use of the known IGF-II receptor blocker, mannose-6-phosphate (see section 5.2). Continuous addition of M-6-P to fibroblast cultures that were initially treated with TGF- $\beta 1$ resulted in an approximately 40% decrease in myofibroblast numbers at day 14 (but no significant decrease between days 0 and 7) compared to the cultures treated by TGF- $\beta 1$ alone (see fig. 5.2.1b). This demonstrates that M-6-P does not significantly inhibit the effect of the added activated TGF- $\beta 1$ but does inhibit the activation of autocrine TGF- $\beta 1$ by the IGF-II receptor. Addition of insulin (plus TGF- $\beta 1$ for three days) to similar cultures for only three days achieved the same level of reduction in myofibroblast numbers and with a very similar pattern over time as the addition of continuous M-6-P achieved (see figs. 5.2.1a and b). Furthermore, addition of insulin and M-6-P to the cultures did not produce an additive affect in terms of inhibition of myofibroblast numbers (see fig. 5.2.1c). The implication therefore is that insulin inhibits the activation of autocrine TGF- $\beta 1$ by the IGF-II receptor just as does M-6-P does. This does not of course mean that insulin binds the IGF-II receptor in the same way that M-6-P does, but simply that

some level of interaction, direct or indirect exists between insulin and the IGF-II receptor. This could represent a future (commercial) challenge for any insulin-derived anti-scarring therapy as M-6-P is currently entering the clinical trial stage of development (Ferguson et al 2004). On the other hand, insulin requires a once-only dosing to achieve the same effect as continuous M-6-P which is clearly much more suited to clinical applications. This difference reflects their difference in their mechanisms of action: whilst M-6-P is a ligand of the IGF-II receptor, insulin is not (under physiological conditions at least) (Bräulke et al. 1989).

If insulin does inhibit the activation of autocrine TGF- β by some sort of interaction with the IGF-II receptor then we might expect to see some effect on the levels of latent TGF- β in terms of either the large or small latent complexes. Immunostaining of latent TGF- β in fibroblast cultures (see figs. 5.4.1 and 5.4.2) suggests that there may be higher levels of the inactivated latent TGF- β in insulin-treated cells. In contrast Western blotting revealed no significant change in the levels of active TGF- β (25 kD band) (figs. 5.3.3-4) present although this varied considerably between cell strains and also between different repeat experiments, thus making the quantification of active TGF- β using this methodology is inconclusive. However although Western blotting (see figs. 5.3.5-7) did not reveal a significant change in the protein levels of either the SLC or LLC it did suggest a trend towards higher LLC levels in insulin treated fibroblasts. If this were to prove to be a real trend then it would imply that insulin is inhibiting the activation of autocrine TGF- β 1 in terms of the LTBP being cleaved from the SLC. Precisely how the LLC is activated is unknown but may not necessarily

involve the IGF-II receptor (Miyazono et al. 1991). A further interesting trend (not reaching significance) was revealed by analysis of the ratios of the ODs of the TGF- β 1 and SLC bands (fig. 5.3.6) which revealed this ratio was 36% less in the insulin treated cells. This hints at a decrease in activation of TGF- β 1 rather than changes in its absolute level of expression.

This last trend received further supportive evidence from the highly sensitive TGF- β bioactivity assay developed by Rifkin's group (Abe et al., 1994) (see section 5.4). This showed that the addition of insulin to fibroblast cultures led to an approximately 50% reduction in the proportion of active (autocrine) TGF- β in the cultures (see figs. 5.4.1 and 2). This significant finding explains many of the other results namely that insulin doesn't affect the expression of TGF- β 1 (see chapter 4 and figs. 5.3.5-8) but appears to produce effects on myofibroblast differentiation that are similar to that of M-6-P (fig. 5.3.1) and will not inhibit myofibroblast differentiation when active TGF- β is present but will inhibit its delayed effects after its removed (fig. 4.5.1).

There is clear evidence that insulin does appear to inhibit the activation of latent TGF- β , giving a similar net end result to that of M-6-P treatment. The exact mechanism of insulin's action however is unclear but is likely to differ from that of the M-6-P as it does not bind the IGF-II receptor. Insulin cannot act as a competitive inhibitor of the IGF-II receptor in the same way as M-6-P does. Nonetheless, there are other ways insulin could act to interfere with this receptor's actions such as affecting its ligand or affinity or its expression and cellular distribution. Immunostaining studies of the IGF-II

receptor showed a trend towards reduced proportions of those fibroblasts strongly expressing the receptor (see figs. 5.6.1 and 2), RT-PCR did not indicate any significant decrease in mRNA expression with insulin treatment (see fig. 5.6.3), but Western blotting did suggest a trend of reduced IGF-II receptor protein expression in insulin-treated fibroblasts (see figs. 5.6.4 and 5) although this did not reach significance. These last results regarding the IGF-II receptor are unfortunately rather inconclusive. This could be due to the high variability observed between cell lines. Repetition of the experiment with greater numbers of cell lines may resolve this issue, however this was not possible due to time constraints. Another potentially fruitful line of enquiry would be to determine changes in receptor affinity for latent TGF- β using radio-labelled ligand binding studies.

However, there are other sources of activation of autocrine TGF- β 1 apart from those described above such as plasmin, other proteases and thrombospondin (Daniel et al. 2004). On the other hand, these alternative mechanisms may only have minor roles physiologically and in any case as figure 5.2.1 demonstrates inhibition of the IGF-II receptor with M-6-P considerably reduces myofibroblast differentiation.

The exact nature of insulin's effect on the IGF-II receptor remains unclear.

Redistribution of the receptor has been proposed but there is no compelling evidence and other research has shown an increase rather than a decrease in IGF-II receptor numbers at the cell surface in response to insulin treatment (Bräulke et al, 1989) although this is accompanied by decreased phosphorylation of the receptors (Covera et

al, 1988). Although 80-90% of the IGF-II receptors are actually on the internal membranes (Braulke et al. 1987) an equilibrium exists with the surface receptors and any internal changes in receptor concentration could well affect the secretion or retention of receptor ligands such as IGF-II (Braulke et al. 1989). Future studies may clarify insulin's relationship with the IGF-II receptor. For example, FACS analysis (Roederer 2002) or radiolabelling of ligands to quantify the receptors on the cell surface.

7.1.7 Potential sources of error in the laboratory techniques

Assessment of myofibroblast numbers throughout this research has been by counting cells visualised under the microscope. This introduces some degree of error because the intensity of the α -SMA staining varies making identification of myofibroblasts sometimes difficult. Random selection of triplicate fields was used to ameliorate these difficulties but had the side-effect of limiting the number of experiments that could practically be performed and for each experiment close to 2000 cells have been counted for each cell line. Perhaps in the future a computer software package that is sophisticated enough to allow the counting of both intensely staining and faintly staining myofibroblasts will enable this process to be automated. Expression of α -SMA can be quantifiable via Western blotting or alternatively with a system described by Tanaka et al (Tanaka, Sano et al 2001), using a novel cell capture enzyme immuno assay. The problem with both techniques is that α -SMA expression varies between cells and the presence of rare highly positive cells can disproportionately increase the level

of α -SMA seen. A small proportion of these extremely bright α -SMA positive cells are always present in fibroblast cultures.

Related to the potential inaccuracy of myofibroblast counting is the question of how safe is the assumption that fibroblasts expressing α -SMA are true myofibroblasts. The effects of insulin may not necessarily be specific to myofibroblasts but specific to α -SMA; that is, insulin may decrease the expression of α -SMA but not necessarily prevent the differentiation of myofibroblasts altogether. The experiments (see chapter 3) that demonstrated that insulin did not significantly affect the expression of stress fibres, EDA fibronectin and FAK kinase for example, raise this possibility. A further assumption is that a decrease in the expression of α -SMA equates to better scarring; this can only really be answered with further clinical trials in which the clinical appearance of scars is compared with their α -SMA content. More precise identification of myofibroblasts would require electron microscopy to visualise of structures like the fibronexus (Aidan 2001). However, this would clearly be impractical and the widespread application of α -SMA-immunostaining to identify myofibroblasts throughout the literature indicates its broad acceptance.

It has also been assumed that the reduction in myofibroblast numbers seen with insulin treatment is due to inhibition of myofibroblast differentiation rather than apoptosis of existing myofibroblasts. This is known to happen during wound healing (Desmouliere et al. 2005) and scar maturation (Vande Berg JS et al, 1985). However, this would lead

to a reduction of myofibroblast numbers below those seen in the controls and this was not observed.

The question of the accuracy of α -SMA immunostaining is relevant to the whole thesis. This research has frequently employed immunostaining to judge the relative levels of expression of different proteins in different cultures. Unfortunately this technique requires the relevant protein to be in sufficiently high levels in the cells and accessible to its antibody if the staining is to be detectable. Clearly this may not be the case for every protein examined in this thesis and furthermore the judgement of 'positivity' with immunostaining is invariably to some degree subjective. Of course, corroborating evidence from immunostaining with Western blotting is ideal but this has proved in the case of some proteins to be not technically feasible or practical within the limits of time available. Furthermore, other directions in the research proved more promising and so efforts were directed instead towards those.

7.1.8 The Clinical Trial

Preparation of the clinical trial began with determining the most suitable commercially available formulation of insulin to use. Previous work by Mackie (2004) had indicated that the medium length acting Insulatard[®] inhibited myofibroblast differentiation in vitro at lower doses than shorter acting formulations. Results presented in this thesis further showed that Insulatard[®] inhibits myofibroblast differentiation in vitro at a lower dose than insulin glargine (a long-acting insulin with no peak of activity) (see fig. 6.2.1). This suggests that the peak of insulin activity or availability is more important

than its duration of activity or availability with respect to myofibroblast differentiation. Nevertheless it is hard to make any meaningful judgements as the insulin formulations were designed to perform in situ subcutaneously. However, their pharmacokinetic properties are likely to be very different when applied in media surrounding the cells. Insulatard[®] was chosen for use in the clinical trial due to its reliable efficacy in vitro and in the murine in vivo model. Although Insulatard[®] proved to be the most efficacious formulation in vitro, this does not necessarily mean it will be the most efficacious in vivo. This should be tested in future trials. Both formulations of insulin had their maximal effect at supraphysiological concentrations (physiological levels are 6 -26µIU/ml (Entwistle 1990), maximum effect in experiment at 0.5-1.0IU/ml) indicating that insulin's actions are unlikely to be solely due to the insulin receptors (because these would be already saturated) and insulin does not bind the IGF-II receptor at any concentration. Although insulin is known to bind to the IGF-I receptor at high concentrations, its involvement is considered unlikely due to previous work by Linge et al. that showed that IGF-I itself did not share the myofibroblast inhibitory effect of insulin.

Due to the difficulty of objectively comparing scars several new ways of assessing scars were included in the trial. These enabled multiple characteristics of a scar to be measured with relatively little expense apart from multiphoton microscopy. All of these techniques are subject to errors but in combination it would seem reasonable that they would detect any significant improvement in scar formation. Reassuringly the different

methods did agree on which scars were 'good' and which were 'poor'. These methods could be re-employed for a further trial.

Generally speaking statistical analysis of the placebo-treated group versus the insulin-treated group for all the different methods of assessing the scars failed to reach statistical significance. Indeed the panel assessment of photographs of the scars yielded significantly improved scar scale scores on the insulin-treated side in only 6 patients, with 5 patients showing significant improvement on the placebo-treated sides and 4 patients showing no significant difference (see fig. 6.5.5). This remained true if just the VAS scores were considered (see fig. 6.5.6). Nevertheless, in 9 out of 15 patients the insulin-treated scar was lower in volume (see fig. 6.5.1). Overall the insulin-treated scars displayed a trend of being less luminous by 20% ($P = 0.378$) (see fig. 6.5.10) and less red ($P = 0.16$) (see fig. 6.5.9) in the treated scars. The remaining parameters that were assessed namely scar prominence and width, did not show any significant difference between the two groups.

The findings from the laboratory research suggested that the clinical trial might produce more convincing results. However, the in vitro environment is a much more controlled and invariable environment. In the in vivo environment there is much more variation and an array of cytokines and hormones that may oppose insulin's action in addition to the circulation which redistributes and metabolises the insulin. This could be challenged in future trials by using a variety of (higher) doses and in larger numbers of patients. It is also possible that using the lateralmost ends of the inframammary wounds

could mean substantial variation in the amount of tension or distortion of the skin thus affecting scarring. A better model might be to use an intra-wound control during scar revision surgery for instance.

The clinical trial demonstrated insulin's potential to meet the criteria for an ideal antiscarring agent listed in the introduction to this chapter (section 7.1). The £12 vials containing 10mls of 100 units per ml of insulin that were used in this trial would each be enough to treat the full breast reduction scars (about 50cm per patient) of dozens of patients. The safety of insulin has been well tested and the trial confirmed that its use in this application was not associated with any adverse effects and in particular did not make scarring worse or delay wound healing and is of course non-allergenic. Insulin was administered in very dilute form via syringe and needle, a method that is widely available, cheap and suitable for all scars perhaps with the exception of burns. The murine in vivo model (Mackie 2004) demonstrated insulin's greatest efficacy was with a single dose, and this is a quality that could be further tested in a larger clinical trial. The benefits of single dosing in clinical terms are multiple. Firstly, it avoids repeated painful needle exposures which for children may mean repeated general anaesthetics; secondly, it means the patient does not have to return to clinic for further treatments which is particularly helpful in developing countries where patients may have to travel large distances, and lastly it is more cost and time effective.

The trial was designed to mimic as closely as possible the experiments in the mice described by Mackie (2004). Dosing is clearly critical to the effectiveness of any drug

and could have been the most important factor in the outcome of the trial. In the trial the dose administered was simply doubled to reflect the doubling of wound length going from mouse to human. Yet, in contrast with the trial findings, in the murine model the insulin treated scars virtually disappeared. This could be due to the intrinsic differences in the skin of mice and humans such as the facts that scarring in the mouse is known to be very good and with no incidence of hypertrophic scarring. But it could also be that the doses are not equivalent as the murine dermis is much thinner than the human's and therefore the dose was relatively higher in the mouse. In addition, although the same dose was administered to each patient, the actual tissue concentrations may have varied considerably between individuals due to variations in depth of infiltration, proximity of large vessels and individual differences in skin vascularity. Thus shortly after injection the local concentration would have been supraphysiological but the rate at which this drops would vary between individuals, perhaps to a significant degree. Therefore, the agent may have been absorbed too quickly in some individuals to properly exert its effect. This potential problem could be partially circumvented by higher doses of insulin formulations of differing pharmacokinetic properties.

The limitations of having a trial with relatively small numbers was compounded by the fact that most of the patients developed very good scars on the placebo-treated sides. This made it difficult to detect any differences between treated and control scars. Although 4 patients did develop hypertrophic scars, insulin did not appear to be efficacious in inhibiting hypertrophic scar formation. Insulin may improve normal scars

although not dramatically. Nevertheless, this conclusion is helpful in directing future laboratory (such as the use of normal scar cell lines and investigating the mechanism of normal rather than hypertrophic scar formation) and clinical studies (that look specifically at normal scars).

7.2 Conclusion

The work presented in this thesis confirms the myofibroblast inhibitory effects of insulin. As for insulin's possible mechanism of action, no evidence has been found to suggest roles for EDA-fibronectin, stress fibres and other molecules such as Thy-1 and thrombospondin. This research reiterates the powerful role of TGF- β 1 in scarring but also reinforces the impression that TGF- β 2 and 3 play little role in normal adult scarring. However, evidence has been presented to suggest that the activation of autocrine TGF- β 1 is inhibited by insulin and that the IGF-II receptor may be involved in this mechanism although in what way exactly remains unclear.

The clinical trial was relatively inconclusive and is in keeping with the findings of other trials in that normal scarring is very resistant to treatment and pathological scarring remains challenging. The novel methods of scar assessment described, particularly in relation to digital image analysis and silicone moulds add cheap and easy techniques to the existing selection. However, the findings of this study do suggest that further trials with greater numbers of participants and longer follow-up periods are worth pursuing.

In conclusion, insulin demonstrates efficacy in the inhibition of myofibroblast differentiation in vitro, its mechanism appears to involve the inhibition of the activation of autocrine TGF- β 1 and the first clinical trial of insulin as an antiscarring agent has proved inconclusive.

Appendices

Appendix I

Is the expression of the housekeeping gene GAPDH effected by insulin?

I.1 Introduction

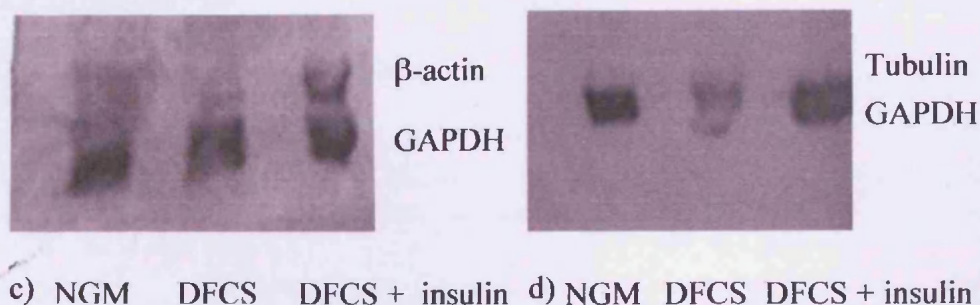
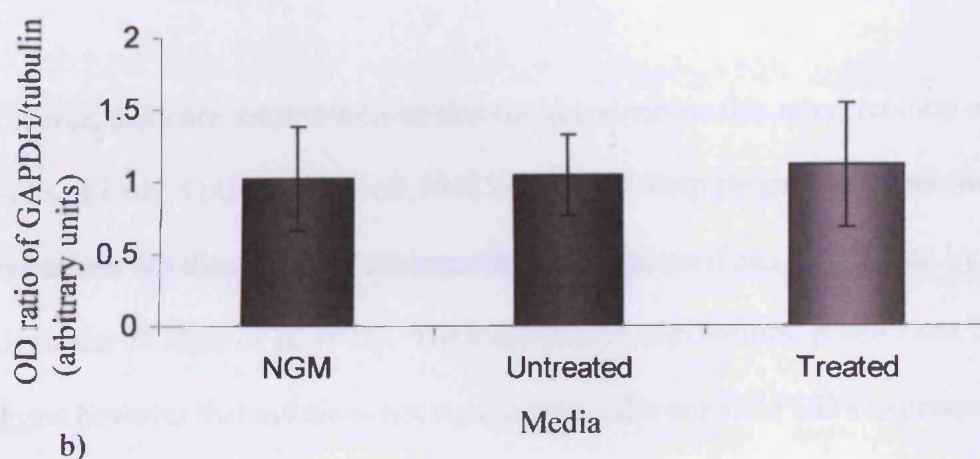
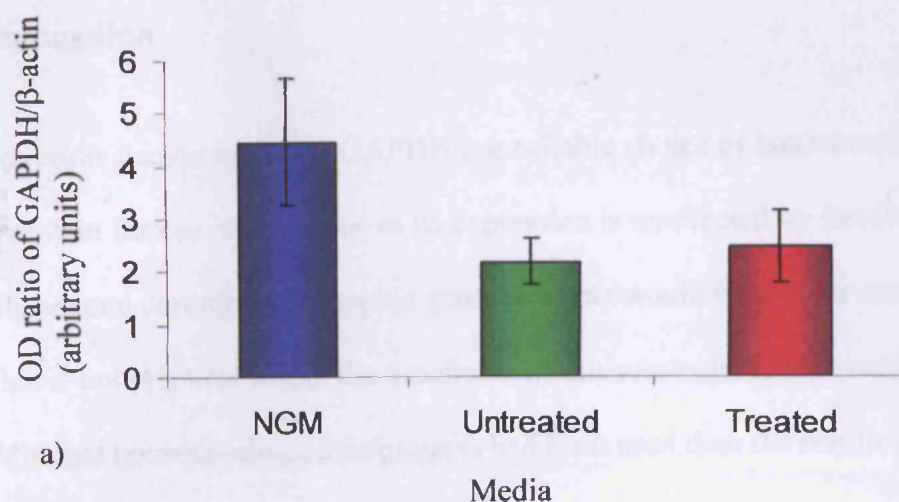
Throughout this thesis, GAPDH has been used as the housekeeping gene (Barber et al. 2005) for comparison with the expression of a variety of substances in both PCR and Western blotting. However, it is possible that the mRNA or protein expression of GAPDH itself is effected by insulin. This would make interpretation of results so far very difficult, hence the need to compare GAPDH's expression with other housekeeping mRNAs/proteins with and without exposure to insulin. Tubulin and β -actin are two other ubiquitously expressed housekeeping genes commonly used for both PCR and Western blotting (Ferguson et al. 2005). Tubulin is a protein that forms part of the components of cellular microtubules whilst β -actin forms part of actin chains. In repeated studies they have been found to be stable, reliable controls at the mRNA and protein levels although with some discrepancies between different tissues (Ferguson et al. 2005). By comparing the expression by normal scar fibroblasts of GAPDH with each of these proteins in the presence and absence of insulin, it will be possible to tell whether GAPDH is affected by insulin and therefore whether it is a suitable control.

I.2 Method

Normal scar fibroblasts were cultured in DFCS with and without insulin (5µg/ml) for 14 days in T75 flasks. The cells at day 0 numbered 7.5×10^5 and by day 14 were near confluence. Media was refreshed twice weekly. The protein lysate was prepared from the flasks as previously described (see section 2.5.1). Western blot analysis was performed with anti-GAPDH (R & D Systems), anti- β -actin (R & D Systems) and anti-tubulin antibodies.

I.3 Results

The results (see figures I.1 a-d) show that the protein expression of GAPDH compared to the housekeeping proteins β -actin and tubulin does not vary in the presence of insulin treatment.



Figs. I.1 a-d. Densitometry analysis of Western blots for GAPDH and a) β -actin and b) tubulin taken from normal scar fibroblasts cultured in DFCS with and without insulin ($5\mu\text{g/ml}$) for 14 days. $N = 3$ cell strains. Error bars represent SEM. No significant difference in GAPDH expression is seen with insulin treatment compared with either β -actin or tubulin. c) and d) are representative blots for c) β -actin (43 kDa) and GAPDH (37kDa) and d) tubulin (50-55 kDa) and GAPDH.

1.4 Discussion

These results demonstrate that GAPDH is a suitable choice of housekeeping gene/protein for use in this thesis as its expression is unaffected by insulin treatment. Ideally several control housekeeping genes/proteins would be used in each experiment but this is not practical within the timeframe of this research. These results suggest that if additional housekeeping genes/proteins had been used then the results so far would probably not have been any different.

However, there are some criticisms that could undermine this interpretation of the results. Firstly, GAPDH has been used as the housekeeping gene and protein for comparison but there is some evidence that GAPDH itself can be affected by insulin (Alexander-Bridges et al. 1992). The experiments with tubulin, β -actin and GAPDH suggest however that insulin is not significantly affecting GAPDH's expression in relation to these other two constitutively expressed housekeeping genes.

Appendix II

Patient information sheet for clinical trial



Department of Plastic Surgery and Burns

Tel:

Patient Information Sheet

An investigation into a novel clinical use of a single low dose of insulin in the prevention of excessive cutaneous scarring

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

Scarring is the normal result of wound healing following operations, traumatic wounds and burns. However, scars can often be unsightly and psychologically disabling and some may impair the normal function of the body. Abnormal scars can also develop which outgrow the original wound and become very disfiguring.

At the RAFT Institute (Restoration of Appearance and Function Trust) that is attached to Mount Vernon Hospital researchers have found that a small once-only dose of insulin administered to a wound at the time of surgery may reduce the severity of scarring following an operation. This study aims to show whether insulin can reduce scarring in patients having routine surgery.

Why have I been chosen?

You have been chosen as a potential patient to be in the study because you are having bilateral breast reduction surgery. This means that you will have two identical wounds on both sides of the body, only one of which will be treated with insulin. This means that we can compare the scars on each side. The study will involve fifteen patients.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

If you take part in the study your operation, dressings, number and timing of clinic visits will all remain exactly the same as if you had not taken part. You will not need any additional blood tests or x-rays. The only extra is that at these clinic visits your scars will be examined and photos and moulds taken of the scars. These photos will be kept confidential and viewed only by researchers and will be for the purpose of comparing the scars on a computer. The moulds will be used to measure the prominence of the scars.

As is usual after this type of operative procedure, you will attend clinic two to three times to see your surgical team and the last visit is usually three months after surgery. This will be the end of your participation in the study.

This study is what scientists call a double blind study. This means that although one of your breast wounds will receive treatment, neither you, your surgeon or the researcher will know which one until the end of the study. This is to prevent any bias when we are recording the results. Nevertheless, if your surgeon did need to know which breast had received treatment, he or she would be able to find out straightaway.

The breast that does not receive the treatment will instead be administered a placebo, in this case a small volume of saline (sterile salty water).

What do I have to do?

If you do take part in the study we would ask you to not apply any creams or lotions (herbal, homeopathic or pharmaceutical) to your scars after the operation as this may confuse the results. There are no other special requirements other than those relating to the operation itself. There are no dietary restrictions. You can continue to take your regular medication. You need not refrain from giving blood and there is no effect on pregnancy.

What is the drug or procedure that is being tested?

Insulin is a naturally occurring substance in the body that has a wide range of effects including the control of blood sugar. The doses used for this study (0.3 IU) are so small that they do not have any effects on the rest of the body or the blood sugar levels. Furthermore, the insulin used is not manufactured from either animals or humans and lasts for up to 36 hours. The insulin is administered at the end of the operation while you are still anaesthetised and is via an injection to the edges of a 3 cm length of the wound.

What are the alternatives for diagnosis or treatment?

There are currently no other scar prevention treatments.

What are the side effects of any treatment received when taking part?

There may be some temporary swelling or inflammation at the injection site.

What are the possible disadvantages and risks of taking part?

There may be some temporary swelling or inflammation at the injection site. If the insulin treatment is successful, the treated part of the scar may be less noticeable than the rest of the scar and the scar on the other side. Thus there may be some asymmetry. However, as only 3cm is treated only a fraction of the total length of the scar is affected. Furthermore, these scars tend to fade with time anyway so that the asymmetry would become less noticeable.

What are the possible benefits of taking part?

We hope that this treatment reduces the scarring from your operation. However, this cannot be guaranteed. The information we get from this study may help us to reduce scarring in future patients.

What if something goes wrong?

We do not expect anything to go wrong as a direct result of the study. If however you are harmed in this study due to someone's negligence, then you may have grounds for a legal action for which you would need to pay. Regardless of this, if you have any cause to complain about any aspect of the way you have been approached or treated during the course of this study, the normal NHS complaints mechanism is available to you.

Formal complaints should be addressed to the *Complaints Department, Hemel Hempstead General Hospital*, (Tel:).

Should you require independent advice about making a complaint or seeking compensation, you may wish to contact the Independent Complaints Advocacy Service (ICAS) for Bedfordshire & Hertfordshire:

Tel:

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. This may involve examining your medical records. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

Your GP and surgical team will be informed of your participation in the trial.

What will happen to the results of the research study?

The results of the study are likely to be published in two to three years in a medical journal. You can obtain a copy of the published results by writing to RAFT at the address below. You will not be identifiable from the publication nor from any photographs of your scars used in the publication.

Who is organising and funding the research?

This research study is funded by charitable donations from individuals, companies and charities.

The doctor conducting this research is not being paid for undertaking this research or including you in this study.

Who has reviewed the study?

This research study has been reviewed and approved by the East & North Hertfordshire Hospitals Local Research Ethics Committee.

Contact for Further Information

Mr Richard Baker, Research Fellow

Tel:

Your help in taking part in this study is invaluable and we are very grateful for your contribution.

You will be given a copy of this information sheet and a signed consent form to keep.

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